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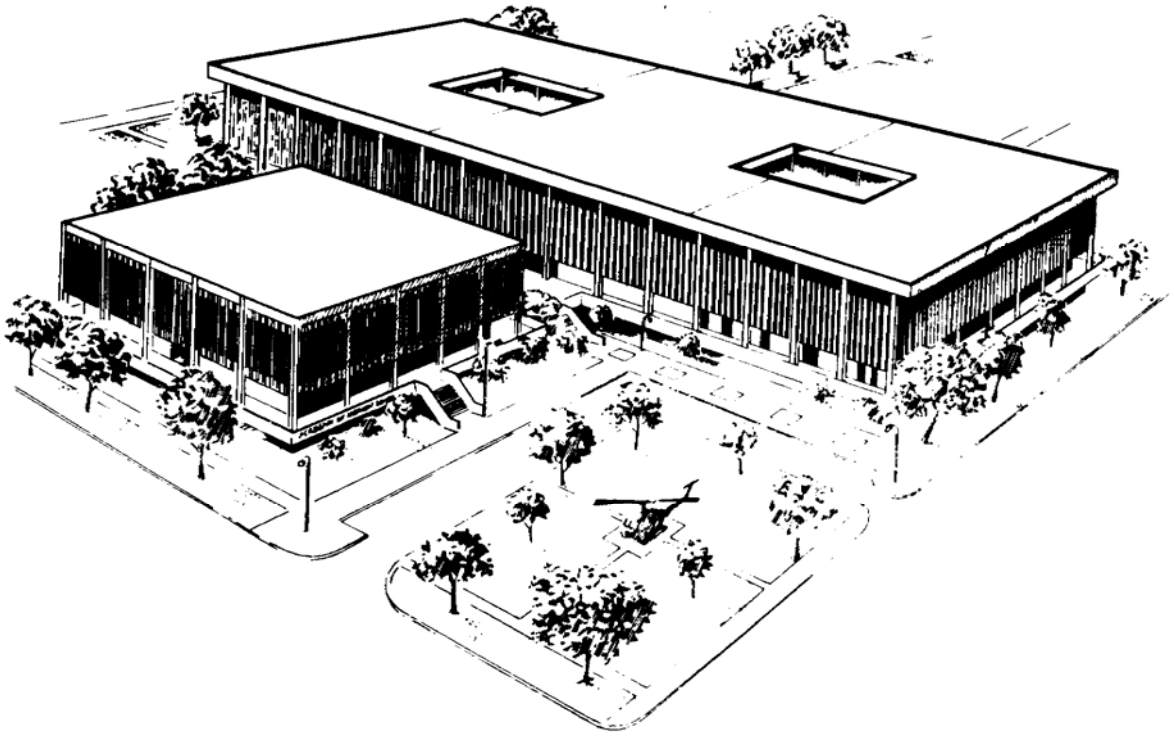

IMMUNOHEMATOLOGY AND BLOOD BANKING II

SUBCOURSE MD0846 EDITION 100

DEVELOPMENT

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CORRESPONDENCE COURSE OF
THE U.S. ARMY MEDICAL DEPARTMENT CENTER AND SCHOOL

SUBCOURSE MD0846

IMMUNOHEMATOLOGY AND BLOOD BANKING II

INTRODUCTION

Blood for transfusion is a biologically active therapeutic substance. It has specific effects on the human organism and dosage requirements, just as any other therapeutic substance. Blood differs, however, from other biologicals in that it must be obtained from healthy individuals of the human race. At our present state of knowledge, human whole blood is a perishable substance, and being derived from human beings, must be collected in thousands of widely spread laboratories, rather than being produced in a handful of qualified and well regulated laboratories. The responsibilities of those who collect, process, and issue whole blood for transfusion are tremendous.

Blood transfusion attempts are recorded as far back as the 16th century. At first, animal blood was transfused to humans. Later, human blood was used. However, facts concerning the characteristics of even human blood were not known, and these early transfusions frequently caused severe reactions, and often death.

It can be readily seen that immunohematology is a relatively new medical area, if one stops to consider that Landsteiner discovered three of the four blood groups in the ABO system in 1900. A year later the fourth blood group was discovered, but it was not until 1937 that the Rh factor was discovered. Since that time, tremendous strides have been made in the field of hematology and blood banking.

The safety of blood transfusion is directly proportional to the knowledge, skill, and sense of responsibility of the laboratory technicians. It is not sufficient to establish appropriate procedures and techniques. Continuous instruction and training are required, to assure highest quality and safety in performance of blood bank procedures. That is why, in this subcourse, instruction is concerned with immunohematology, the newborn, and blood transfusion for such.

<u>Subcourse Components:</u>

This subcourse consists of three lessons and an appendix.

Lesson 1, Immunohematology.
Lesson 2, Immune Hemolytic Anemia and Hemolytic Disease of the Newborn.
Lesson 3, Blood Transfusion.
Appendix, Bibliography.

Credit Awarded:

Upon successful completion of the examination for this subcourse, you will be awarded 14 credit hours.

To receive credit hours, you must be officially enrolled and complete an examination furnished by the Nonresident Instruction Branch at Fort Sam Houston, Texas.

You can enroll by going to the web site http://atrrs.army.mil and enrolling under "Self Development" (School Code 555).

A listing of correspondence courses and subcourses available through the Nonresident Instruction Section is found in Chapter 4 of DA Pamphlet 350-59, Army Correspondence Course Program Catalog. The DA PAM is available at the following website: http://www.usapa.army.mil/pdffiles/p350-59.pdf.

LESSON ASSIGNMENT

LESSON 1	Immunohematology
TEXT ASSIGNMENT	Paragraph 1-1 through 1-53.
LESSON OBJECTIVES	After completing this lesson, you should be able to:

1-1. Identify the factors in the immune response and the interaction of antigens, antibodies, and complement of immunohematology.

1-2. Identify the applications of and sources of error in the antiglobulin tests.

1-3. Identify the features of the antibody screening test.

1-4. Identify the factors in the use of reagent red cell panels to identify unexpected antibodies, both cold and warm.

1-5. Identify the factors in the titration of antibodies.

1-6. Define absorption and elution.

1-7. Identify the aspects of compatibility testing, collection of blood from the transfusion recipient, selection of blood for a recipient, compatibility testing for a massive transfusion, the one-tube and two-tube techniques for cross-matching, and the investigation of incompatible crossmatches.

SUGGESTION After completing the assignment, complete the exercises at the end of this lesson. These exercises will help you to achieve the lesson objectives.

LESSON 1

IMMUNOHEMATOLOGY

Section I. THE IMMUNE RESPONSE AND THE INTERACTION OF ANTIGENS, ANTIBODIES, AND COMPLEMENT IN IMMUNOHEMATOLOGY

1-1. BACKGROUND

Immunology, a field once dominated by bacteriologists, has become important to scientists in many other areas. The field of immunohematology came into being when Landsteiner discovered that the blood antigens (ABO) present on RBCs (RBCs) would react with their respective antibodies present in plasma, and that this reaction had great clinical significance. Since that time, many discoveries in this field have added to the understanding of immune mechanisms operative in health and disease. It is important that scientists working in areas associated with blood transfusion understand basic immunology and try to be familiar with the recent advances in this field that might relate directly to their work.

1-2. THE IMMUME RESPONSE

According to Roitt, the basic of immunology is memory, specificity, and the recognition of "nonself". The original basis for this was the protection (immunity) afforded by exposure to infectious illness. The first contact with an infectious organism imprints some information (for example, memory) so that the body will recognize and attack that organism when it encounters it in the future. The protection is usually specific (for example, only against the original infecting organism). The body also has to recognize that organism as being foreign (that is., "nonself"). The substance initially responsible for an immune response is known as an antigen or more specifically an immunogen.

1-3. ANTIGENS

a. Antigens are substances that can induce a specific immunologic response or can interact with specific antibody or immune cells "in vivo" or "in vitro". The immune response can be either humoral or cellular (paragraph 1-4). Blood group serology is mainly concerned with the humoral response that leads to the production of free antibody in the plasma. The antibodies, under appropriate conditions of reaction (temperature, pH, ionic strength, and so forth.), will react specifically with the antigen in some observable way (for example., agglutination, hemolysis).

b. An antigen contains structural chemical groups in a specific three-dimensional arrangement, known as antigenic determinants (epitopes), which are lacking or foreign to the immunized animal. Each antigen can contain many of these epitopes. The specific three-dimensional shape of these antigenic determinants, or chemical groupings, is what determines the specificity of its reaction with a particular antibody molecule.

c. An Important factor affecting the immunogenicity of an antigen is its molecular size. Immunogenic molecules are rarely less than 4,000 daltons. Much smaller molecules (for example, drugs such as penicillin) can be immunogenic if coupled to a protein "carrier" of larger molecular weight. Such a molecule is termed a hapten and can be defined as a small molecule that, by itself, cannot stimulate antibody synthesis but will combine with antibody once formed. Indeed, most of our basic understanding of antigen specificity came from work by Landsteiner using haptens.

d. Blood group antigens are chemical groupings present on the RBC membrane. We are only just beginning to learn the exact nature of these determinants. The ABH antigens have been the most thoroughly studied and when present on RBCs are predominantly glycolipids. A and B antigens are composed of the same fatty acids and sugars, the difference in specificity being caused by the terminal sugar in the chain of sugars joined to the fatty acid backbone. The specificity is a result not only of the particular sugar but also the configuration of the end grouping it forms. As the sugars responsible for A or B specificity (N-acetylgalactosamine and galactose, respectively) are structurally identical except for the substitution of an hydroxyl group for an N-acetylamino group at carbon atom number two, they serve as a good example of the remarkable specificity of antigen-antibody reactions.

e. Proteins are direct gene products, whereas carbohydrates, such as the A and B antigens, are indirect products of genes (for example, A or B genes). The direct (protein) products of the A and B genes are enzymes that recognize and then transfer specific sugars from their nucleotide carriers to specific acceptor molecules. Thus, the A gene product is an N-acetyl-D-galactos-aminyltransferase and the B gene product in a D-galactosyltransferase.

f. The biologic role of blood group antigens, if any, is at present unknown. The ABH antigens are widely distributed throughout the body, being present on many types of cells, organs, and body fluids. Some antigens such as Rh and Kell (K) appear to play a part in cell membrane integrity. Rare individuals lacking Rh antigens (Rh_{null}) on their RBCs often have an associated hemolytic anemia ("Rh-null syndrome"), whereas, in contrast, rare individuals lacking A, B, and H antigens (Bombay phenotype) do not. It has been suggested that this is because the ABH antigens are glycolipids projecting above the cell membrane, whereas Rh appears to be lipoprotein, an integral part of the RBC membrane. An association between a rare inherited defect of neutrophil bactericidal function (chronic granulomatous disease) and the Kell blood group system has recently been described. Another report suggests a possible relationship between the Duffy blood group antigens and resistance to malaria. There are many other associations of blood groups with disease, particularly malignancy; many of them are purely statistical and their causes unknown.

1-4. ANTIBODY SYNTHESIS

a. **The Process of Antibody Synthesis.** When an antigen enters the body, it may evoke a humoral response, in which antibodies are synthesized by plasma cells and released into the body fluids (for example, plasma), and/or a cellular response, in which lymphocytes participate in cell-mediated immunity (for example, rejection of transplanted tissue and delayed hypersensitivity). That two different responses were present was originally shown by Chase and Landsteiner in the early 1940s when they demonstrated that some kinds of immune reaction could be transferred from one animal to another by the exchange of living cells, whereas others could be transferred by blood serum. The cells required for the former experiment were lymphocytes. It was not until the early 1960s that involvement of the lymphocyte was proven.

b. **Lymphocyte Populations.** Stem cells from the bone marrow are thought to differentiate to form two distinct lymphocyte populations. The cells that pass through the thymus become known as T-lymphocytes (T-cells) and the others that are independent of the thymus B-lymphocytes (B-cells). Although these lymphocytes look similar by conventional light or electron microscopy, they do look very different by scanning electron microscopy, and also they can be differentiated by a variety of surface markers. Their functions are of course different, but there is mounting evidence for the possibility of cooperation between the two systems.

c. **T-Lymphocytes.** Once leaving the thymus, where they are known as thymocytes, the T-lymphocytes are immunocompetent, that is to say, capable of participating in an immune response. This is the basis of cellular immunity. T-lymphocytes constitute the greater part of the recirculating pool of small lymphocytes and have a relatively long half-life. When they encounter an antigen (which may have to be first processed by a macrophage), they transform to lymphoblasts (See figure 1-1). These T-lymphoblasts, which have no demonstrable intracellular immunoglobulin, have several functions:

(1) They divide further into primed antigen-sensitive cells, which provide immunologic memory because of their long life span.

(2) They release a number of soluble factors (lymphokines) which mediate delayed-type hypersensitivity.

(3) They are “killer” cells, which are cytotoxic for cells bearing the histo-compatibility antigens of a graft or tumor cells.

(4) They may cooperate with the humoral system by triggering B-lymphocytes.

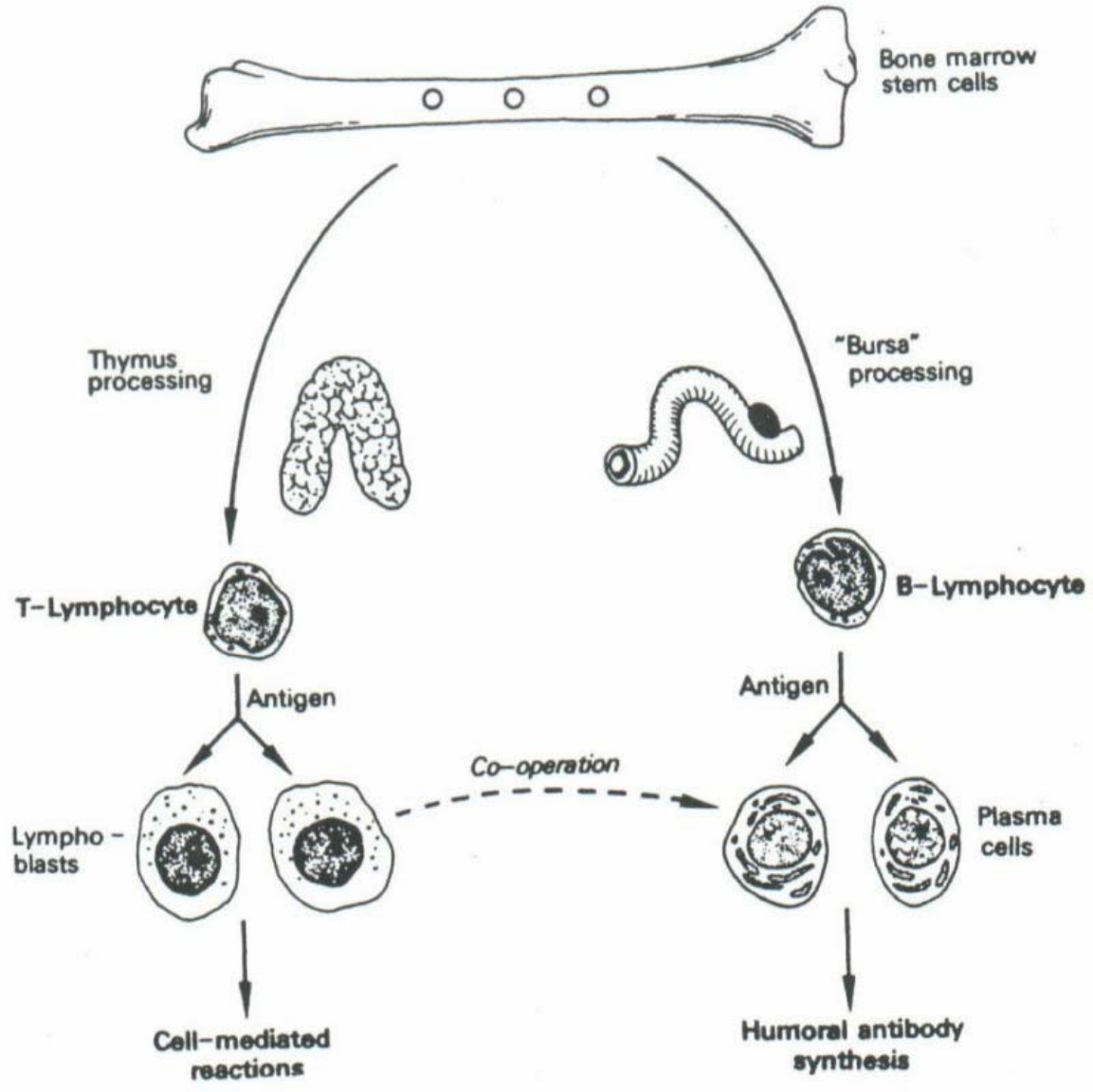


Figure 1-1. Processing of bone marrow cells by thymus and gut-associated lymphoid tissue become immunocompetent T- and B-lymphocytes, respectively. Proliferation and transformation of the lymphoblast and plasma cell series occurs on antigenic stimulation (From Roitt).

d. **B-Lymphocytes.** The B-lymphocyte gets its name from the Bursa of Fabricius, a lymphoid organ present in birds, which controls the production of lymphocytes responsible for making humoral antibody. The equivalent organ in man has yet to be found. Thymus-independent, or B-lymphocytes synthesize and excrete specific antibodies (surface immunoglobulins) that serve as receptors for antigens. When triggered by antigen, the B-lymphocytes change to plasma cells, which are responsible for the excretion of free antibody into the body fluids (for example, humoral antibody), see figure 1-1. There is much evidence to suggest that macrophages are required to process antigens for appropriate presentation to lymphocytes before the humoral response occurs. In addition, many antigens appear to require the cooperation of both B- and T-lymphocytes. The mechanisms by which T- and B-lymphocytes interact are complex and far from clear at present. As mentioned previously, it is humoral antibodies that are dealt with routinely in blood transfusion science, but possibly cellular reactions will increase in importance in the future.

e. **Differentiation of T- and B-Lymphocytes.** Approximately 25 percent of human blood lymphocytes are B cells, 70 percent T cells, and 5 percent have neither T nor B markers (they are called “null cells”). Immunoglobulins are readily demonstrable on B, but not T-lymphocytes by immunofluorescence. T- but not B-lymphocytes will form "spontaneous” rosettes with unsensitized sheep erythrocytes.

1-5. PRIMARY AND SECONDARY IMMUNE RESPONSES

a. Following a first exposure to a foreign antigen, specific antibodies can appear after about five days, rise slowly to a modest level, remain for a variable period, then gradually decline, eventually becoming undetectable, until further stimulation occurs. The first antibodies produced in this primary response are usually lgM, but eventually other immunoglobulins (for example, lgG) may appear. The type of antigen and the route of administration will influence the pattern observed.

b. After the primary response, a second dose of the same antigen, given days or even years later, will usually elicit an intense and accelerated secondary (memory) response. The serum antibody usually begins to rise within two or three days, reaching a peak in about 10 days. In this secondary response, lgM antibody is often transiently produced, following a similar pattern to the primary response, but the predominant antibody produced is lgG, which rises to a much greater concentration than the lgM, and remains in the plasma much longer. The secondary response is sometimes called an anamnestic response.

1-6. IMMUNIZATION TO BLOOD-GROUP ANTIGENS

a. Within a few months after birth, an infant makes anti-A and/or anti-B, if lacking those antigens on its RBCs. Such antibodies are termed naturally occurring since they have no apparent antigenic stimulus. Experiments in chicks have shown that these antibodies probably develop as a result of exposure to bacterial antigens, closely related chemically to blood group antigens (for example, Escherichia coli has an antigen on its membrane closely resembling human B antigen). Naturally occurring antibodies to antigens other than ABO are also often encountered, particularly in the I, Lewis, P, and MN systems. These antibodies are usually IgM and react better at lower temperatures.

b. Immune antibodies to blood group antigens usually develop as a result of pregnancy, transfusion, or immunization (intential sensitization). Following immunization, IgM antibodies are often seen first, followed by IgG antibodies, which often predominate. These antibodies usually react better at 37ºC.

c. Antibodies other than anti-A or anti-B are usually called “irregular", "atypical", or "unexpected” antibodies. The preferred term is unexpected.

d. There is extensive evidence in animals, such as mice, that the immune response is genetically controlled (by the so-called Ir genes). It has been suggested that this may apply in man also. Studies in man on the immune response to the Rh (D) antigen indicate that approximately 30 percent of the Rh_o(D)-negative individuals appear to be incapable of forming anti-Rh_o(D) even after repeated and/or large transfusions of Rh_o(D)-positive blood. The antibody response in individuals who do make antibody will depend on many factors, including the relative potency of the antigen, the route of immunization, and the amount of blood given.

1-7. ANTIBODY STRUCTURE, FUNCTION, AND PROPERTIES

a. Plasma proteins with antibody activity are called immunoglobulins (Ig). During the last ten years, great advances have been made in defining their structure, physiochemical properties, antigenic characteristics, serologic behavior, and biological properties.

b. Each immunoglobulin molecule consists of basic units, each composed of four polypeptide chains, two light chains (L) and two heavy chains (H), held together by covalent disulfide bonds (S-S), and noncovalent interactions (see figure 1-2).

c. Five classes of immunoglobulins have been recognized on the basis of antigenic differences in the heavy chain: IgG, IgA, IgM, IgD, and IgE (see figure 1-3). No blood groups antibodies have yet been found to be IgD or IgE. There are two types of light chains (kappa chain and lambda chain), which are common to, and found in, all five immunoglobulin classes, but each individual immunoglobulin molecule has only one type of light chain. Approximately 66 percent of the molecules of each class have kappa light chains, and 33 percent have lambda light chains.

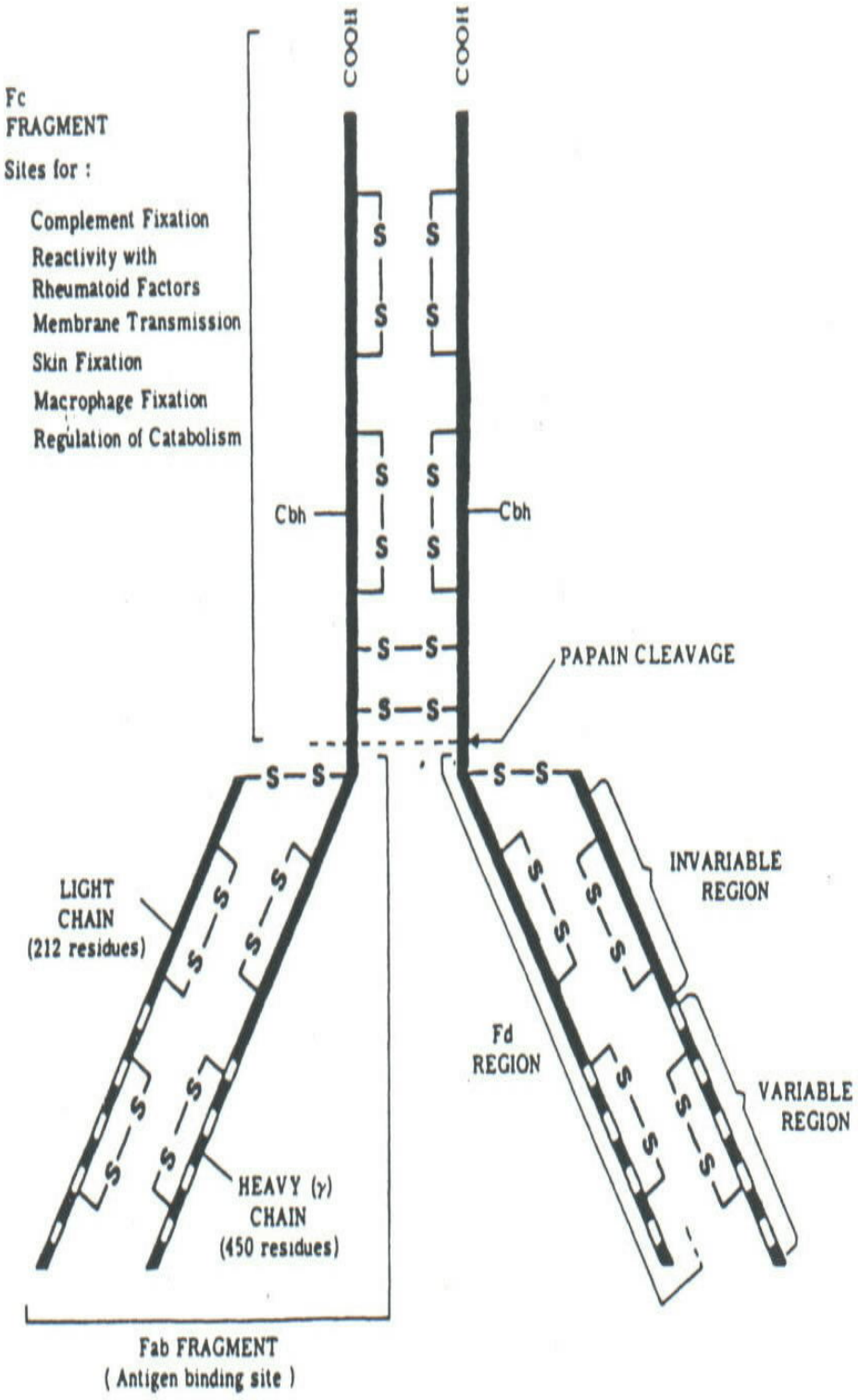


Figure 1-2. The four-chain structure of an IgG molecule showing both interchain and intrachain disulfide bridges. (Cbh = carbohydrate.

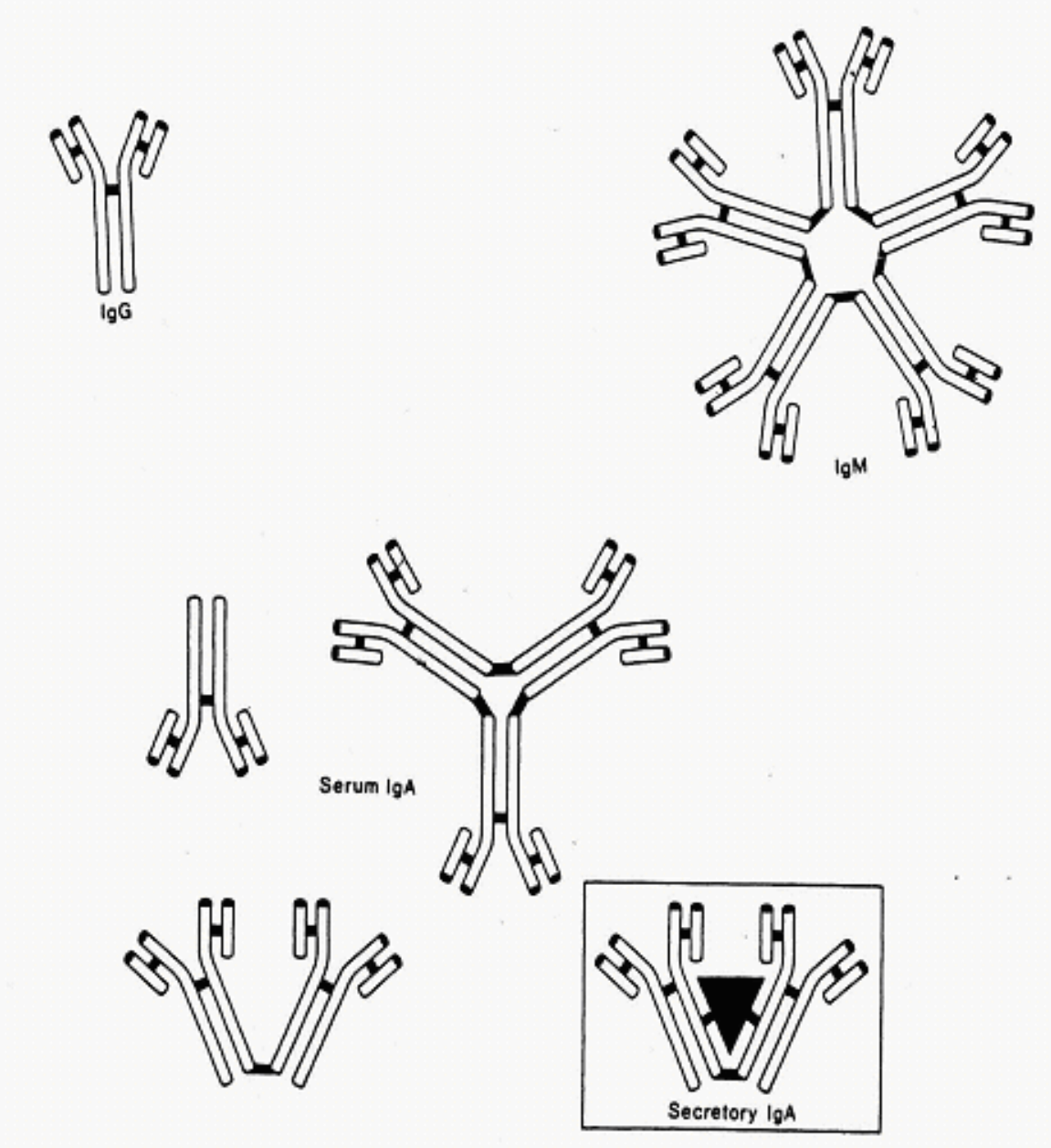


Figure 1-3. Comparative structures of antibody molecules that have been shown to have blood group activity. IgG is shown as a simplemonomer. IgM is seen as a pentamer. Serum IgA can be found as a monomer, dimer, or trimer. Secretory IgA is seen as a dimer to which a secretory piece is added.

d. The determination of the amino acid sequence, of both light and heavy immunglobulin polypeptide chains, show that these chains can be divided into two domains. A light chain has two such domains, one variable (V_L) and one constant (C_L). An lgG heavy chain has one variable (V_H), and three constant (C_H1, C_H2, CH3) domains. The variable domains confer specificity on the antibody molecule, and two of them; one from the light and one from the heavy chain, form the binding site for antigen (see figure 1-3). The constant region of both heavy and light chains imparts the class and subclass characteristics to the immunoglobulin molecule.

e. Papain cleavage of the molecule yields three fragments. Two, composed of part H chain and part L chain (Fab fragments), retain the antigen-binding properties of the whole antibody molecule, whereas, a third, the Fc fragment, is concerned with a variety of general biologic functions such as complement fixation, placental transfer, and skin sensitization (see figure. 1-2).

f. On the basis of antigenic and functional differences, each immununoglobulin class can be shown to consist of subclasses. In the case of lgG, four such subclasses are recognized: lgG1, lgG2, lgG3, and lgG4. The various immunoglobulin classes, although primarily identified by differences In the amino acid composition of the constant region of their heavy chains, demonstrate differences in a matter of other properties such as: physico-chemical properties (electrophoresis, sedimentation, carbohydrate composition); antigenic properties; biologic properties (skin-sensitizing properties, ability to cross the placenta, complement fixation, and so forth); and serologic behavior. The types of immununoglobulins and their properties are summarized in Table 1-1.

Class (WHO nomenclature, (1964)	**IgG** **G**	**IgA** **A**	**IgM** **M**	**IgD** **D**	**IgE** **E**
Old nomenclature	2 SS, 7S-	A_1 2^A	1M, 2^A 19S		
Structure					
H-chain, class	4	2	2	2?	?
Number subclasses					
L-chain, types	150,000	180,000 - 500,000	900,000	180,000	200,000
Molecular weight					
Electrophorectic mobility		slow	between	between	fast
Sedimentation constant $^{S}20^{W}$	6.7	7-15	19	7	8
Carbohydrate percentage	2.6	5-10	9.8	10 - 12	11
Gm allotypes (H-chain)	+	0	0	0	0
Inv allotypes (L-chain)	+	+	?	?	?
Concentration, mg per 100 ml	1,000-1500	200-350	85-205	3.0	0.01-0.07
Secretion	0	+	0	0	0
Antibody activity	Yes	Yes	Yes	?	Yes reagent
Serologic characteristics	Usually incomplete	Usually complete	Usually complete	?	?
Fix complement	Yes	No	Yes	Yes	Yes
Crosses placenta	Yes	No	No	No	No

Table 1-1. Human Immunoglobulins.

1-8. ANTIGEN-ANTIBODY REACTIONS IN BLOOD GROUP SEROLOGY

a. Antibodies may react with their specific antigens in a number of ways. The following reactions have all been used to demonstrate “in vitro” antigen-antibody reactions in blood transfusion science:

(1) Agglutination.

(2) Hemolysis.

(3) Inhibition.

(4) Absorption and elution.

(5) Precipitation.

(6) Complement-fixation.

(7) Radioimmunoassay.

(8) Fluorescence.

b. The first two methods are the most commonly used in blood group serology and will be discussed in more detail. Inhibition and absorption/elution techniques, although not used every day in the routine blood bank, are used regularly in the forensic laboratory (for example, blood grouping of blood stains) and in reference laboratories. Absorption techniques lead to a decrease in antibody activity following treatment of a serum with RBCs having the appropriate antigens; elution refers to the technique used to dissociate or remove antibody bound to sensitized RBCs. Precipitation, complement-fixation, and radioimmunoassay have been utilized more in blood banks in the last few years, particularly for the detection of hepatitis virus. Fluorescence has been used to demonstrate blood group antigens (for example, ABH) in tissues.

1-9. AGGLUTINATION

a. **Background.** It is convenient to consider antibody-mediated agglutination of RBCs as involving two distinct stages. First, there is physical attachment of antibody to the antigenic determinant on the RBC surface. This stage, representing the specific immunochemical reaction, is referred to as sensitization. It may go on to involve the binding or fixing of complement components. The second stage involves agglutination of the sensitized cells. Agglutination results from collision of sensitized cells, allowing cross-linking of cells to occur by the formation of antibody bridges. As the aim of blood group serology is to obtain maximum sensitivity without loss of specificity, it is important to understand and recognize the factors that influence the complex agglutination phenomenon.

b. **Factors Affecting the First Stage (Sensitization).** Red blood cell sensitization with antibody obeys the law of mass action. Thus, the reaction between antigen on the RBC surface and antibody is reversible and the quantity of cell-bound antibody at equilibrium will vary depending on the reaction conditions and the equilibrium constant of the antibody. The reaction conditions should be designed to maximize the quantity of cell-bound antibody at equilibrium in order to facilitate detection of either blood group antigen or antibody. Some of these reaction conditions are described below:

(1) Temperature. Most blood group antibodies show their greatest reactivity over a restricted temperature range, some reacting optimally at 4ºC, others at 37ºC. Antibodies reacting optimally at 37ºC have been described as “warm” antibodies, and those reacting optimally at lower temperatures as “cold” antibodies. Agglutinins (antibodies) having maximum reactivity at one temperature may have sufficient thermal amplitude to be active at others. Antibody activity is usually tested at room temperature and at 37ºC. Antibodies active at 37ºC are the most clinically significant, although “cold” antibodies cannot be ignored if they have a wide thermal amplitude (for example, above 30ºC). Antibodies only reacting at lower temperatures may be of importance in patients subjected to hypothermia.

(2) pH. The pH optima for antibody reactivity in most blood-group systems have not been investigated. For anti-Rh_O(D), the optimum pH lies between 6.5 and 7. Antibodies of other blood-group specificities may have different pH optima (for example, some examples of anti-M react best at pH 5.5).

(3) Incubation time. Time is required for the antibody RBC reaction to reach equilibrium. The amount of time required to reach this state will depend upon other variables. The rate of antibody binding is greatest initially, so incubation times for routine laboratory procedures may be relatively short (for example, 15 to 30 minutes).

(4) Ionic strength.

(a) The ionic strength of the reaction medium is one of the physiochemical conditions that play an important role in the binding of antibody to RBC antigens. Ionic strength is a measure of intensity of the electrical field resulting from ions in solution. Electro-static forces (interaction of positive and negative charges) play an important role in antibody reaction involving RBCs. Red blood cells carry a large electronegative charge, which serves to keep them from spontaneously aggregating. This enables them to function efficiently in oxygen transport by maintaining a maximum surface area available for gas diffusion. When RBCs are suspended in an electrolyte solution (0.85 percent NaCl), the cations (positive) are attracted to the negatively charged RBCs, and the RBC becomes surrounded by a diffuse double layer (“ionic cloud”), that travels with the RBC as if it were part of it. The outer edge of this layer is called the surface of shear or the

slipping plane. The effective charge (potential) of the RBC, called the zeta potential, is determined at this plane and is responsible for the electrostatic repulsion between one RBC and another.

(b) In the first stage of agglutination, reducing the ionic strength of the medium decreases the electropositive clouds of cations surrounding the RBCs and facilitates the interaction of electropositive lgG with the negatively charged RBC. This absorption of antibody to the RBC reduces the electronegative charge of the RBC and reduces the zeta potential, thereby accelerating the second stage. Experiments have shown that the initial rate of association of anti-Rh_O(D) with Rh_O(D)-positive RBCs is increased 1,000-fold by a reduction of ionic strength from 0.17 to 0.03 (for example, instead of using 0.9 percent NaCl, 0.2 percent NaCl in 7 percent glucose or 0.3M glycine is used as a RBC diluent).

(5) Antigen-antibody ratio. The rate at which antibody is bound to the cell, and the quantity of antibody bound, depend on the concentration of cells and of antibody. In general, an increase in sensitivity is obtained by increasing the amount of antibody in relation to antigen. This is often achieved in the blood bank by using less antigen in the form of weaker cell suspensions (for example, it is a more sensitive technique to add one volume of two percent RBCs to two volumes of serum, than to add one volume of ten percent RBCs to two volumes of serum). Some agglutination reactions are weakened or even become negative in the presence of an excess of antibody, the prozone reactions phenomenon. The optimal proportion of antigen to commercial antiserum is usually determined by the manufacturer; the directions issued with each antiserum should be followed.

c. **The Second Stage (Agglutination).**

(1) Once RBCs are sensitized, they may or may not directly agglutinate. Blood group antibodies were characterized empirically before the immunoglobulin classes were recognized. Those antibodies that could produce agglutination in a saline medium were called “complete” antibodies or ‘bivalent” antibodies, and those that did not were called "incomplete" antibodies or “univalent” antibodies. Current evidence indicates that all antibodies are at least bivalent; that is, each molecule has at least two antigen-combining sites. The term incomplete antibody is used to denote an antibody that reacts with, but fails to cause visible agglutination of a saline suspension of RBCs possessing the corresponding antigenic determinant; such antibodies tend to be of class lgG.

(2) The failure of “incomplete" antibodies to produce agglutination in a saline environment may be a result, in part, of location, number, and mobility of antigenic determinants on the RBC surface, of the size and configuration of the antibody molecule, and of the electrostatic forces involved.

(3) It has been suggested that the zeta potential, mentioned previously, is the most important factor In explaining why most lgG antibodies do not directly agglutinate RBCs, the span of the lgG molecules not being sufficient to bridge adjacent RBCs under the conditions created by the electrostatic forces keeping the RBCs apart. The same workers suggested that lgG antibodies agglutinated RBCs in the presence of albumin because albumin raises the dielectric constant (charge dissipating power) of the suspending medium, thus lowering the zeta potential, allowing RBCs to come close enough together for agglutination to occur. They also suggested that proteolytic enzymes (for example, papain, ficin, bromelin, and trypsin) produce the same final effect by cleaving sialic acid from the RBC membrane, thus reducing the zeta potential. It should be noted that lgG antibodies (for example, lgG anti-A and -B) do sometimes directly agglutinate saline-suspended RBCs; this may be a result of the large number of antigenic sites present, the orientation of these antigens above the surface of the RBC membrane, and/or the clustering of these antigens during the antigen-antibody interaction. Recently some workers have argued that zeta potential may not be the most important factor involved in these reactions.

1-10. HEMOLYSIS

Some blood group antibodies can activate the complement cascade (see paragraph 1-11), leading to lysis of RBCs possessing the appropriate antigens. Antibodies showing this characteristic are termed hemolysins and usually will agglutinate or sensitize RBCs in the absence of complement. Examples of blood-group antibodies that can sometimes act as hemolysins are anti-A, -B, -A, B, -I, -i, -Le^a, -Le^b, -Le^x, -Jk^a, -Jk^b, -PP_1P^k(TJ^a), -Vel. Some of these antibodies and others may sensitize the RBCs with complement, but not hemolyze them. This complement sensitization can be detected by the antiglobulin test.

1-11. COMPLEMENT

a. The complex complement system is involved in the humoral portions of the inflammatory response arid interacts broadly with portions of the clotting sequence, the fibrinolytic system and the kinin-generating sequence. The function of complement appears to be activation of secondary immunologic actions resulting in cytolysis, phagocytosis, chemotaxis, and so forth The complement system consists of at least 20 globulins, many exerting their effect by enzymatic activity. The components are numbered C1 to C9. They contribute about 5 percent of the total plasma proteins. Most of them are beta globulins and are good immunogens. C3 is by far the most abundant complement protein (1,500 :g/ml) and C4 the next at 450 :g/ml; most of the other components being present only in small amounts. It is C3 and C4 that most interest us in Immunohematology, particularly in reference to the antiglobulin test.

b. Two major pathways of complement activation have been identified, the classic pathway and the alternative pathway. The classic pathway is of most importance in blood transfusion science.

1-12. THE CLASSIC COMPLEMENT PATHWAY

a. Activation of the classic pathway can be initiated by a number of substances, the best known of these, and probably the most important, being the immunoglobulin molecule. Only one molecule of IgM on the cell membrane is necessary to activate the complement system (two subunits of the IgM molecule combining with adjacent antigens on the membrane). In contrast, it is thought that IgG needs to form a "doublet"; that is to say, two separate IgG molecules have to combine with adjacent antigens on the cell membrane as close together as 250 to 400 A, before they are able to activate C1. Only certain subclasses of IgG are able to activate complement through this pathway. IgG3 is the most efficient, followed by IgG1; IgG2 is the least efficient. IgG4 does not activate the complement; neither does IgA.

b. Appropriate interaction of antibody with antigen leads to sequential activation of the complement system, often ending in cytolysis. This involves a series of protein-protein interactions resulting in the generation of a series of cellular intermediates bearing successively bound complement components. An antibody-sensitized erythrocyte is designated EA, and successive complement components are designated by numbers, for example, EACI, EAC1, 4. The protein components of complement circulate in the plasma in an inactive state, and once activated are designated by a bar over the component number, for example, C1. The activation process usually is achieved by cleavage of the next complement molecule into fragments, which are designated by lower case letters, for example, C3a, C3b. The activated products usually have enzymatic properties; thus the whole pathway is an enzymatic cascade similar to the coagulation cascade (see figure 1-4). The system is held in check by the instability of the complexes formed and the naturally occurring inhibitors and inactivators present in normal plasma (for example, C3b INA).

c. The pathway consists of three operationally defined functional units, the recognition unit (C1), the activation unit (C4, C2, C3), and the membrane attack unit (C5, C6, C7, C8, C9).

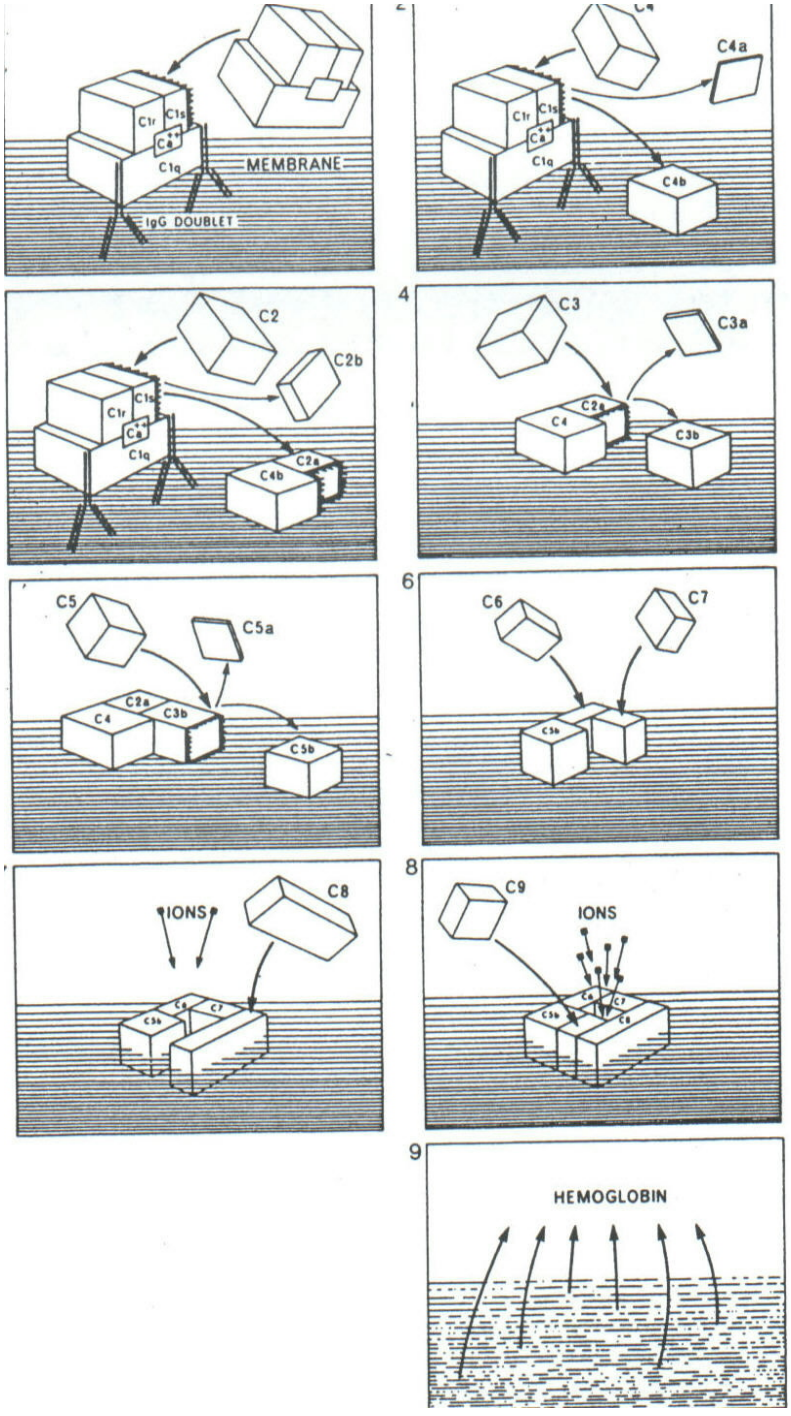
(1) Recognition unit. C1 is a complex of three proteins held together by calcium. C1q is a collagen-like protein with binding sites for IgG and gM; C1r is the activating enzyme of the critical catalytic site of the C1 complex, C1s is a proenzyme, activated by C1r. When C1 collides with an antigen-antibody complex (EA), it is bound to the Fc fragment of the immunoglobulin molecule through the C1q subunit. This activates C1r and subsequently C1s by cleavage of a single polypeptide chain.

(2) Activation unit. This unit is assembled in two stages. Activated C1(C1s) acts on native C4 by cleaving the molecule into C4a and C4b. The major fragment C4b attaches to the cell membrane. A shower of fragments is produced by a single CIs enzyme, so that many C4b molecules may cluster around the EAC1 site on the cell. C1s also cleaves native C2 into two fragments; the major C2 fragment, C2a, combines with C4b on the cell membrane to form an active complex C4b2a (C3 convertase), that

has enzymatic activity directed against C3. Magnesium ions are necessary for the formation of the C4b2a complex. C3 is cleaved by the C4b2a complex into two molecules, C3a and C3b. The smaller C3a (MW 10,000) does not bind to the cell membrane, but is released into the fluid phase as a mediator of inflammation (anaphylatoxin I). The C3b molecule (MW 175,000) binds to the cell membrane and can also bind to its own activation enzyme. As the C4b2a complex is an enzyme, it can react more than once, and produce a shower of C3b fragments each time. Only the C3b fragments that become bound adjacent to the C4b2a enzyme, however, are believed to participate in the next reaction, in which C5 is cleaved.

(3) Membrane attack unit. Some of the C3b molecules combine with C4b2a to form C4b, 2a, and 3b, which will cleave the C5 molecule into C5a (anaphylatoxin II) and C5b. This is the last enzymatic reaction in the pathway. C5b appears to bind C6 and C7 by absorption. The resulting trimolecular complex attaches to the cell membrane and binds C8 and C9. Fully assembled, the membrane attack complex consists of one molecule of C5b, C6, C7, C8, and up to six molecules of C9. It has a molecular weight of about one million. The end result of the pathway is lysis of the cell (see figure 1-4).

d. Electron microscopy shows that lesions start appearing in the cell membrane after C8 is absorbed, although the cell does not lyse until C9 is complexed. It is not understood how these lesions are made. In most instances, the lesions are not large enough to allow the hemoglobin molecule to escape directly through the lesion, so it is thought that cell lysis is caused by an osmotic effect. When cells are attacked by complement, they swell until the cell membrane is ruptured. The cause of the swelling is salt and water entering the cell. Mayer has postulated a theory he calls his “doughnut” hypothesis: a stable hole is produced by the assembly of a rigid, doughnut-shaped structure in the lipid bilayer of the cell membrane. The hole forms a channel connecting the inside of the cell with the extracellular fluid. The outside of the doughnut could be composed of nonpolar polypeptides, that is, protein chains that were hydrophobic; the interior would need polar peptides so that it could be hydrophilic. He suggests that C5b, C6, C7, C8, and C9 may be the proteins that form the doughnut or funnel shape, penetrating the lipid bilayer of the membrane.



1-3. Formation of recognition unit. 1. Activation of C1 by an lgG doublet. 2. Step one; activation of C4 by C1. 3. Step two; activation of C2 by C1, to form C4b2a.
4. Cleavage of C3 molecule leading to cell-bound C3b. 5-8. Formation of membrane attack unit; C4b2a3b cleaves C5 molecule. Cell-bound C5b absorbs C6, C7, and C8, leading to membrane defects, thus allowing ions to enter cell. 9. Membrane ruptures releasing hemoglobin from cell. (Courtesy of G. Garratty.)

Figure 1-4. The complement cascade (classic pathway).

1-13. THE ALTERNATIVE COMPLEMENT PATHWAY

a. The alternative pathway bypasses C1, C2, C4, and enters the classic pathway at the C3 stage. It can be activated by several substances including aggregated immunoglobulins and endotoxin. Thus, an antigen-antibody reaction is not necessary to initiate the cascade.

b. This pathway is not fully understood at present, but several factors necessary for its reaction have been isolated. Once C3 is activated in this pathway, the molecular consequences are identical to the classic pathway. The alternative pathway has not been incriminated in many immunohematologic problems yet, but it is of interest to note that the red -blood cells of patients suffering with paroxysmal nocturnal hemoglobinuria (PNH) have been shown to hemolyse through this pathway.

1-14. STABILITY OF COMPLEMENT WITH PARTICULAR REFERENCE TO THE DETECTION OF BLOOD-GROUP ANTIBODIES

a. In order to demonstrate hemolysis or RBC bound complement by the antiglobulin test, one must sensitize RBCs with complement-fixing antibodies in the presence of complement, or in a two-stage technique, in which cells are first incubated with antibody, washed, and then incubated with a source of complement. The most practical source of complement is human serum, but in order to utilize this source, one must have information about the stability of complement under varying conditions of storage. A study by Garratty was specifically designed to determine the effect of storage on the activity of complement with a reference to the detection of blood-group antibodies. In this study, normal serums were stored at -90ºC, -55ºC, -20ºC, 22ºC, and 37ºC from 24 hours to 3 months. Complement activity was assessed by a standard hemolytic assay and also by antiglobulin test assay, which measured the ability of the stored serums to serve as a source of complement in the detection of blood-group antibodies by the antiglobulin test. Hemolytic assays closely paralleled antiglobulin assay. At levels below 60 percent complement activity, there was a danger of missing weak complement-binding antibodies. An average of these assays showed: at 37ºC, activity fell to 30 percent In 24 hours; at room temperature, activity was 40 percent at 48 hours, 8O percent at 24 hours, zero at 72 hours; at 4ºC, as 90 percent at 72 hours and 60 percent at two weeks. At -20ºC, activity was more than 60 percent for 2 months, and at -55ºC and -90ºC, activity was retained at three months.

b. It should be stressed that these studies were carried out on normal serums, and that serums from hospital patients may be deficient in certain complement components before storage, or they may develop anticomplementary properties faster than normal serums. The Standards of the American Association of Blood Banks states that tests for compatibility should be performed on nonactivated, refrigerator-stored serum, collected within 72 hours of performance test.

Section II. THE ANTIGLOBULIN TEST

1-15. PRINCIPLES OF THE ANTIGLOBULIN TEST

a. In 1945, Coombs, Mourant, and Race described a test that detected nonagglutinating (coating) Rh antibodies in serum, and later used the same test to demonstrate “in vivo” coating of RBCs with antibodies. In 1957, Dacie et al. showed that complement components attached to the RBC could also be detected by the test. This test, now known as the antiglobulin test, depends on the following simple principles:

NOTE: “In vivo” means something is measured, seen, or tested inside a living organism. “In vitro” means something is measured, seen, or tested outside a living organism after it has been removed from that organism.

(1) Antibody molecules and complements components are globulins.

(2) If an animal (for example, rabbit or goat) is injected with human globulin (either purified or in whole human serum), the animal will make antibodies to the foreign protein (for example, antihuman globulin, AHG).

(3) This antiglobulin serum, after suitable treatment, will react specifically with human globulin. If this globulin (for example, antibody or complement) is attached to the RBC membrane, the antiglobulin serum will combine with globulin on adjacent RBCs, and cause agglutination of the sensitized RBCs. Nonsensitized RBCs will not react (see figure 1-5).

b. As mentioned in Section I, most blood group antibodies are IgM or IgG. Most IgM antibodies can be detected by direct agglutination; thus, the principal purpose of the antiglobulln test is to detect IgG nonagglutinating (sensitizing) antibodies. In certain circumstances, it may be advantageous to detect RBC-bound IgA, IgM and/or complement components by this test.

c. The antiglobulin test can be used to detect “in vivo” or “in vitro" RBC sensitization.

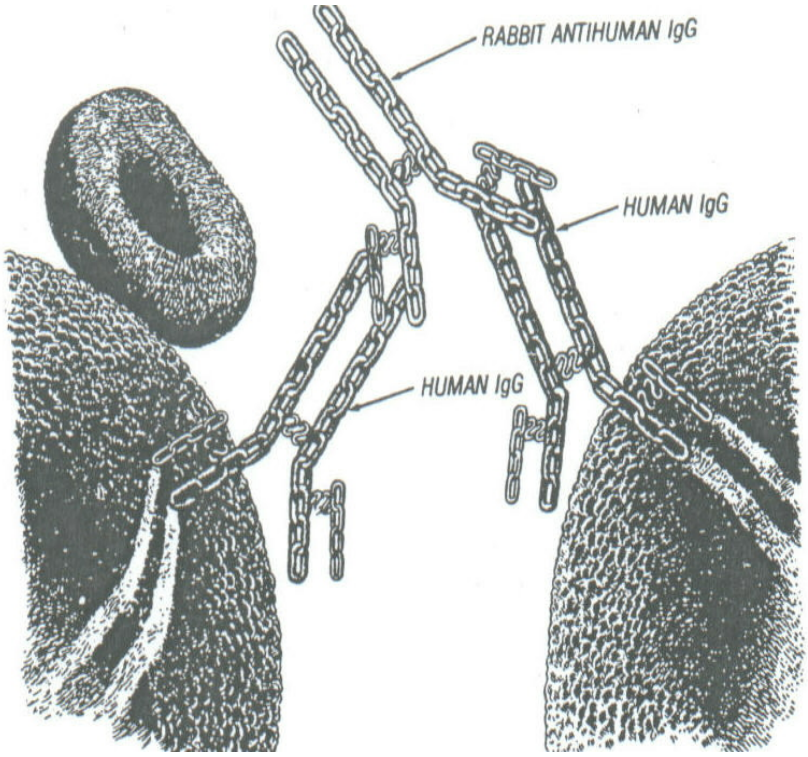


Figure 1-5. The antiglobulin reaction. A rabbit IgG molecule with antihuman IgG specificity is shown reacting with human IgG (for example, anti-Rh_O(D)) on human RBCs. (From Garratty, G.)

1-16. DIRECT ANTIGLOBULIN TEST

NOTE: Below you are given the procedure for the direct antiglobulin test (testing for "in vivo" coating of red cells). For variations of the indirect antiglobulin test (testing for “in vitro” coating of red cells), see the test for D^U in paragraph 1-17, and the two crossmatch procedures on pages found in Section 1-47.

a. The direct antiglobulin test is used for the detection of “in vivo” coating of RBCs with globlins. Washed RBCs from the patient or donor are directly tested with the antiglobulin reagents.

b. The direct antiglobulin test is useful for:

(1) Diagnosis of hemolytic disease of the newborn.

(2) Diagnosis of autoimmune hemolytic anemia.

(3) Investigation of RBC sensitization caused by drugs.

(4) Investigation of transfusion reactions.

c. The procedure for the direct antiglobulin test is presented below.

(1) STEP 1. Place one drop of a 2 to 5 percent saline suspension of cells to tested in a labeled 10-x 75-mm tube. Wash 3 or 4 times with saline. After last wash, decant completely. Add one or two drops of antiglobulin serum: mix.

(2) STEP 2. Centrifuge and examine for agglutination with an optical aid; grade and record results. (The manner in which the RBCs are dislodged from the bottom of the tube is critical. The tube should be held at an angle and shaken gently until all cells are dislodged. Then it should be tilted gently back end forth until an even suspension of cells or agglutinates is observed.)

(3) STEP 3. To control for inadvertent contamination of the antiglobulin serum, add one drop of lgG-sensitized RBCs to any tubes that have been recorded as negative and recentrlfuge. If the patient's cells were washed adequately in the first stage of the test, the control cells should be agglutinated, and the negative result on the patient is valid.

NOTE: If monospecific anticomplement (-C3, -C4 or nongamma) reagents are used (para 1-21), complement-sensitized cells should be substituted for the lgG-sensitized cells in step 3.

1-17. INDIRECT ANT IGLOBULIN TEST

a. The indirect antiglobulin test is used to demonstrate antibodies that may cause RBC sensitization "in vitro". The antibody-containing serum is incubated with specific RBCs, which, following washing, are reacted with antiglobulin serum to see whether RBC sensitization has occurred.

b. The indirect antiglobulin test is useful for:

(1) Detection and identification of unexpected antibodies.

(2) Crossmatching.

(3) Detecting RBC antigens not demonstrable by other techniques.

(4) Special studies, (for example, leukocyte and platelet antibody tests).

c. The technique of the indirect antiglobulin test is included in other sections of this manual where this technique is utilized, (for example, Lesson I, Sections III and IV).

1-18. ROLE OF COMPLEMENT IN THE ANTIGLOBULIN REACTION

Red blood cells can become sensitized with complement components both "in vivo" or "in vitro." One of two main mechanisms is usually involved: (1) complement components may sensitize the RBCs through the action of complement-binding blood group antibody(ies), or (two) immune complexes may activate the complement cascade, causing complement components to attach to RBCs nonspecifically. Whichever of the mechanisms is operative, the end product is the same, in that the RBC becomes sensitized with complement components and may or may not continue to hemolysis. If the RBC does not hemolyse, complement components may still be detected on the RBC membrane by the antiglobulin test. C3 and C4 are the most readily detected, and C3 is the most clinically significant. C3 and C4 are beta globulins (B_{1O} and B_{1E}, respectively); thus, if a RBC is sensitized with C3 or C4, the cell is, in chemical terms, sensitized with beta globulin. When antiglobulin serum is made by injecting a rabbit with human globulin (either fractionated or in human serum), the rabbit will make antibodies to gamma globulin, and also to the "nongamma" globulins, (for example, beta globulin). This anti-non-gamma fraction of the antiglobulin serum containing antibeta globulin will react with the RBC-bound complement (C3 or C4), causing agglutination, a positive antiglobulin test. It is also possible to prepare purified C3 and C4 as immuunogens, so that specific anti-C3 and anti-C4 can be produced.

1-19. MECHANISMS FOR SENSITIZATION OF RED BLOOD CELLS BY COMPLEMENT

a. Activation of Complement by Blood-Group Antibodies.

(1) Some blood group antibodies bind complement to the RBC membrane. Some of them do this very efficiently and commonly cause lysis of normal red cells (for example, anti-A, -B, -Vel, -PP_1P^k(Tj^a); others may sensitize the RBC with complement components, not causing lysis of normal RBCs, unless they are enzyme-treated (for example, anti-Lewis and -Kidd); still, other examples of anti-Lewis, -Kidd, -KeII, -Duffy, -S, -s, -I, -i, -H, and -P_1, may sensitize RBCs with complement components without causing lysis of untreated cells or enzyme-treated cells. It is not understood why some antibodies sensitize RBCs with enough C3 to give strongly positive antiglobulin tests, yet do not proceed to lysis. Some of these antibodies are IgM and cause agglutination as well as complement sensitization of the RBCs (for example, anti-A, -B, -I, -i, and some anti-Lewis). Other, rarer, examples may be IgM and do not cause agglutination under normal conditions (for example, some anti-Lewis, -KeII, -Duffy, and -Kidd); but may sensitize RBCs with complement, detectable by the antiglobulin test. Still others are IgG (for example, some anti-KeII, -Duffy, anti-Kidd); in most cases, the IgG sensitization is readily detected by anti- globulin serum in addition to the complement sensitization, but in some cases (for example, some anti-Kidd), the IgG sensitization is very weak, and the complement sensitization strong.

(2) In most situations where RBCs are sensitized with complement by alloantibodies (for example, “in vitro” or transfused RBCs “in vivo”), the responsible immunoglobulin (for example, IgG) is readily detected by the antiglobulin serum. In certain situations, described below, the immunoglobulin is not readily detectable by routine methods or is not present on the RBC any more.

(a) When lgM antibodies sensitize RBCs without causing direct agglutination, they are very difficult to detect by the anti-globulin test with anti-IgM. The reason for this is unknown. In addition, commercial antiglobulin serums contain very little, if any, anti-lgM. lgM antibodies invariably bind complement and are detected by the anticomplement properties of the antiglobulin serum. This is theoretically a more sensitive method, as every one lgM molecule will cause hundreds of C3 molecules to sensitize the RBC.

(b) About 10 to 20 percent of “warm” autoimmune hemolytic anemias have positive direct antiglobulin tests, resulting from sensitization with complement (coating due to C3 alone), no lgG, lgM or lgA being detected on the RBCs. Some of these patients are thought to have lgG present on their red cells, but it is present in amounts below the threshold of the antiglobulin test as performed routinely.

(c) In cold agglutinin syndrome, the patient’s lgM “cold” autoantibody usually reacts up to 30ºC to 33ºC. Thus, the patient’s RBCs become sensitized with antibody in the peripheral circulation when the skin temperature drops to this range. The antibody usually binds complement to the RBCs, and, if conditions are optimal, hemolysis of the cells occurs. If the cells escape hemolysis, they will recirculate to 37ºC. At 37ºC, the cold autoantibody elutes back from the cell into the plasma, leaving complement components firmly bound to the RBC. Thus, when a positive direct antiglobulin test is obtained on these patients, it is because the anticomplement in the antlglobulin serum has reacted with the RBC-bound complement. No inmunoglobulins are present on the RBC.

b. **Activation of Complement by Immune Complexes.**

(1) Red blood cells can become sensitized with complement because of activation of the complement cascade by immune complexes. Sometimes these immune complexes are attached to the RBC membrane; at other times, they are remote from the cell. A good example of this is the formation of immune complexes involving certain drugs, for example, phenacetin or quinidine. The drug-antidrug complex can attach nonspecifically to RBCs, and cause activation of complement with subsequent attachment of complement compounds to the RBC membrane.

(2) It is important that anticomplement (in particular anti-C3d) activity be present in antiglobulin serums used for direct antiglobulin tests in the diagnosis of autoimmune hemolytic anemia; however, as alloantlbodies detectable only by their ability to bind complement are so rare, the Importance of anticomplement activity in reagents used for compatibility tests is open to debate.

1-20. STANDARDIZATION OF ANTIGLOBULIN SERUMS

a. **Background.**

(1) The most important function of antiglobulin serum is to detect RBC-bound lgG. The present standards used by the Bureau of Biologics (BoB) are in terms of activity against Rh antibodies only (for example, lgG). Most reagents on the market today are prepared and standardized to detect lgG, not only in terms of Rh, but are tested against a wide selection of lgG blood group antibodies. In addition, most of them are prepared and standardized to detect cell-bound complement (in particular C3 and sometimes C4). The presence of other antibodies in the reagents (for example, anti-lgM, -lgA, -albumin, and so forth) is variable, and is usually coincidental, rather than deliberate.

(2) The reagent can be prepared by injecting rabbits with highly purified immunogens, or by harvesting from immuno-secreting whole human serum or protein fractions. Injection of whole serum has several disadvantages, one being that the animal will respond much better to single antigens, than to the many present in whole serum; another being that antibodies to certain proteins (for example, human lgW) show a prozone effect, whereas others (for example, antihuman beta globulin), usually do not. Therefore, it is difficult to select one dilution that detects both lgG and complement optimally. Because of these problems, it is more usual for the animals to be injected with fractions of human serum, either purified proteins (for example, lgG or complement components) or cruder fractions such as gamma and "nongamma" globulin. The commercial houses prepare careful blends from antibodies to the different fractions, and the antlglobulin serum is tested as below. Finally, after being licensed by the FDA, it is distributed as antihuman globulin.

b. **Specificity Testing.** Following a course of immunization, the rabbits are bled, and their serum screened for reactivity against normal non-sensitized RBCs, lgG, and complement-sensitized RBCs. Most of the rabbit serums will contain antispecies, which will have to be removed from the final product, by either fractionation, dilution, or absorption with nonsensitized human RBCs, before it is sold to the consumer.

c. **Antl-lgG Standardization.** Antiglobulln serums are tested against Rh-sensitized RBCs by the so-called checkerboard titration. Rh-positive RBCs are incubated with a series of dilutions of anti-Rh_O(D) to yield RBCs sensitized with lgG, varying from strongly positive to barely positive. Each sample of sensitized cells is tested against each of a range of dilutions prepared from the rabbit anti-globulin serum. In hyperimmunized animals, a prozone is often observed (for example, the more diluted rabbit serum may react better than the less diluted reagent). This means that the weakest sensitized RBCs may react with a 1:200 dilution of the antiglobulin serum, yet not react with lower dilutions (or indeed higher dilutions). Thus, 1:200 would be the optimal dilution of this particular reagent for the detection of Rh (lgG) antibodies. Most commercial houses test their antiglobulin serums against many other lgG antibodies, unfortunately, the optimal dilution for detecting lgG anti-Rh may not be the same for the

optimal detection of IgG anti-Fy^a or Jk^a. Generally speaking, though, the optimal dilutions are similar for most IgG antibodies.

. d. **Anticomplement Standardization.**

(1) The FDA requires that reagents marketed as polyspecific AHG contain anti-C3d activity at a level that equals or exceeds the FDA's anti-C3d reference serum.

(2) When the C3 (B_{10}) molecule is acted on by C3 convertase (C142) in an immune reaction, the molecule is cleaved into two fragments. The smaller C3a fragment does not attach to the RBC membrane, but the larger C3b fragment does.

(3) (Thus, if RBCs are sensitized with complement "in vitro" by a complement binding blood group antibody, they will be sensitized with C3b. They will give a positive antiglobulin test with antiglobulin serums containing antibodies to determinants on the C3b molecule (for example, anti-C3b, anti-C3c [($_{1A}$ or anti-$C3d_{2D}$)]).

(4) When RBCs are sensitized with complement "in vivo" (for example, autoimmune hemolytic anemia or transfusion reactions), or following prolonged incubation "in vitro", the C3b is acted on by the C3 inactivator, which is present in all normal plasma, and the molecule is cleaved into C3c and C3d. The C3c (B_{1A}) fragment is lost from the RBC, and the C3d ($_{2D}$) fragment remains on the cell membrane. (see figure 3-6). Thus, in order to detect C3 bound to the RBC "in vivo", the antiglobulin serum must contain activity against C3d (see figure 1-6).

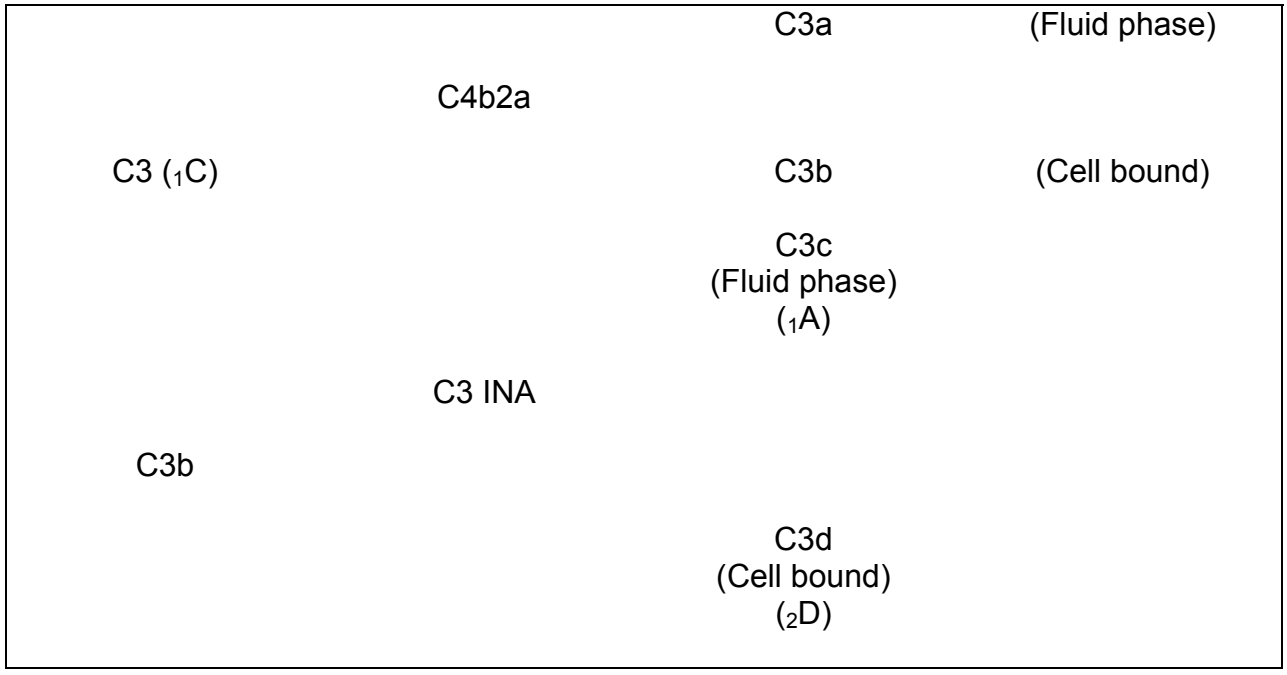


Figure 1-6. Reaction products of C3. (From Garratty and Petz).

(5) Recent published data indicate that C4 is also fragmented in a similar fashion, possibly by the same inactivator. Thus, RBCs sensitized “in vivo” with C4 have only C4d present on their surface.

(6) Generally, the commercial houses standardize their antiglobulin serums against RBCs sensitized with C3, C4, and C3d. The cells are usually sensitized with complement by complement-binding blood group antibodies (for example, anti-Lewis or anti-I) and/or low ionic strength methods. The C3d-sensitized cells can be from patients with autoimmune hemolytic anemia or cells prepared “in vitro”, by treating C3b-sensitized red blood cells with C3 inactivator (for example, in normal serum) or trypsin.

1-21. POLYSPECIFIC ANTIGLOBULIN REAGENTS

a. Polyspecific antiglobulin reagents are used for routine compatibility tests, alloantibody detection, and the DAT. They contain antibody to human IgG and to the C3d component of human complement. Other anticomplement antibodies may be present, including anti-C3d, anti-C4b, and anti-C4d.

b. Since most clinically significant antibodies are IgG, the most important function of polyspecific antiglobulin is to detect the presence of IgG.

1-22. MONOSPECIFIC ANTIGLOBULIN SERUMS

a. As discussed previously, the antiglobulin serum, used routinely in the blood bank for procedures such as compatibility testing, usually reacts with several plasma proteins (particularly IgG and complement). It is possible to prepare monospecific antiglobulin serums by injecting animals with highly purified proteins, such as IgG, IgA, IgM, C3, or C4; or by absorbing unwanted antibodies from the antiglobulin serum, the first method being preferable (See Table 1-2).

b. The main use of monospecific antiglobulin serums is to extend the direct antiglobulin test by evaluating which protein is responsible for the positive direct antiglobulin test obtained with the broad-spectrum reagent (see Lesson 2, Section I). On occasion, monospecific reagents such as anti-IgG and anti-C3 may be of value in the indirect antiglobulin test to show different patterns of reactivity when mixtures of IgG noncomplement-binding and complement-binding antibodies are present (for example, anti-hr$^{"}$ (e) + -Le^{b}). The technique used for testing RBCs with these reagents is exactly the same as that for broad-spectrum reagents.

CAUTION: Never use anti-IgA, -IgM, -C3, -C4 alone for compatibility testing or for antibody detection.

Reagent	Definition
Polyspecific	Contains anti-lgG and anti-C3d; may contain other anticomplement and other anti-immunoglobulin antibodies.
Polyspecific	Contains rabbit antihuman lgG and murine monoclonal anti-C3b and -C3d.
Anti-lgG	Contains anti-lgG with no complement activity.
Anti-lgG (heavy chain)	Contains only antibodies reactive against human gamma chains.
Anti-C3d and Anti-C3b, Anti-C3d	Contains only antibodies reactive against the designated complement component(s), with no anti-immunoglobulin activity.
Anti-C3d (murine monoclonal) Anti-C3b, -C3d (monoclonal)	Contains only antibodies reactive against the designated complement component, with no anti-immunoglobulin activity.

* As defined by the FDA: Code of Federal Regulations 21 CFR 660.

Table 1-2. Antihuman globulin reactions.

1-23. FACTORS AFFECTING THE ANTIGLOBULIN TEST

a. **Sensitization Phase ("In Vitro" Only).**

(1) Temperature. Incubation is normally at 37ºC, as most clinically significant lgG antibodies react optimally at 37ºC. Complement sensitization also occurs optimally at 37ºC.

(2) Medium. The suspending medium for the RBCs may be saline, albumin, or low-ionic-strength saline (LISS) serum. There is evidence that a shorter incubation period can be used if albumin is present in the incubation mixture, and also that uncommon antibodies are detected in the presence of albumin that fail to react when RBCs are suspended in saline. Antibody association is considerably enhanced if RBCs are suspended in a simple low-ionic-strength medium.

(3) Proportions of serum to cells. The same general principles apply here as discussed in paragraph 1-9. Increasing the proportion of antibody to antigen will increase the degree of antibody coating. Two drops of serum to one drop of two percent to five percent RBCs, a proportion of 100:1 to 40:1 in terms of packed RBCs is commonly used. Mollison has shown that by increasing this proportion (for example, to 1000:1), sometimes weak antibodies can be detected that are not detectable in our routine incubation mixtures. In special investigations, such as hemolytic transfusion reaction with no antibody detectable by routine procedures, it might be useful to try increasing the proportion of serum to cells.

(4) Incubation time. A period of 15 to 30 minutes of incubation (especially in the presence of albumin) at 37ºC permits detection of most clinically significant antibodies. Extension of the incubation period to 30 to 60 minutes may detect a few weaker examples of antibodies that are undetected after 15 minutes of incubation.

b. **Washing Phase.**

(1) Washing must be rapid and uninterrupted to minimize loss of cell-bound antibody by elution.

(2) Decant the saline as completely as possible between each washing. Shake to loosen and resuspend the cells completely. Add the saline in a forceful stream. An automatic washer achieves these objectives more efficiently than washing by hand.

(3) Do not cover the mouth of the test tube with the finger, or the palm of the hand, when mixing. Serum remaining on the fingers after handling the specimen can inactivate the antiglobulin reagent.

NOTE: As little as one drop of a 1:4,000 dilution of human serum can neutralize one drop of antiglobulin serum.

(4) Use adequate volumes of saline. When 10- X 75-mm or 12- X 75-mm test tubes are filled at least three-quarters full of saline, three or four washings are usually adequate.

(5) After the final wash, discard the saline as completely as possible. Resuspend the cells and add the appropriate amount of antiglobulin serum. Mix well, and centrifuge. It is important that the antiglobulin serum be added immediately, following completion of washing.

1-24. SOURCES OF ERROR

a. **False Negative Results.**

(1) Inadequate washing of cells will result in neutralization of the antiglobulin serum by trace amounts of residual globulin. A final concentration of only two mg of lgG/ml can cause neutralization of the antiglobulin serum. Thus, the RBCs have to be washed free of unbound IgG, until it is below this figure.

(2) Contamination with human serum will neutralize the reagent. If the reagent dropper is contaminated with serum and replaced in the vial, the entire contents of the vial may be neutralized. If the tube is inverted over the thumb or finger in the washing process, serum contaminating the skin may result in neutralization.

(3) Elution of antibody from the RBCs may take place if the test procedure is interrupted or delayed, particularly during the washing phase.

(4) The optimum temperature for reactivity of the antibody must be maintained during incubation to achieve maximal coating of the cells.

(5) A cell suspension that is too heavy will not permit optimum coating with the antibody; if too weak, reading agglutination may be difficult. A 2 to 5 percent suspension of RBCs is preferred.

(6) Test cells, test serum, and antiglobulin serum lose reactivity if improperly stored.

(7) Some antibodies may be detected only in the presence of active complement. Anticoagulants such as ACD, CPD, or EDTA will chelate calcium, preventing activation of complement. Thus, the use of plasma rather than serum may lead to a false negative reaction. Old or improperly stored serum will also have impaired complement activity.

(8) A prozone reaction should not be a problem with licensed products. Standardization is done by the manufacturer; directions for the test must be followed.

(9) Antiglobulin serum may have been omitted.

(10) Undercentrifugation or overcentrifugation (the latter because of the excessive force needed to resuspend cells).

(11) Insufficient incubation time.

NOTE: The lack of agglutination of presensitized RBCs, added following completion of a negative antiglobulin test, will demonstrate a false-negative determination caused by 1, 2, 6, or 9 above.

b. **False Positive Results.**

(1) Cells having a positive direct antiglobulin test cannot be used with reagent antiserums that require an antiglobulin phase because all such cells will be agglutinated by the antiglobulin serum.

(2) Bacterial contamination of test cells or septicemia in a patient may result in a positive antiglobulin test. If the RBCs are T-activated, they may react as some antglobulin sera contain anti-T.

(3) Extreme reticulocytosis has been reported to give a positive result because of transferrin bound to reticulocytes reacting with antitransferrin in the antiglobulin reagents. Most antiglobulin reagents today have little antitransferrin activity.

(4) Saline stored in glass bottles may contain coilcical silica leached from the container; this has been reported to cause false positive reactions.

(5) Saline stored in metal containers, or used in equipment with metal parts, may contain metallic ions that may bring about nonspecific protein-coating of the RBCs.

(6) Improperly prepared antiglobulin serum may contain traces of species-specific antibodies (This should not be a problem if tests are performed with licensed AHG reagents).

(7) When all the antiglobulin tests are weakly positive, the cause may be improperly cleaned glassware or other form of contamination.

(8) Overcentrifugation may give false-positive results (aggregation vs. agglutination).

(9) Patients' or donors' serums can contain a naturally occurring cold autoantibody (normal incomplete cold antibody) that can sensitize their own or other cells with complement. Usually this only occurs at 4ºC, but it may occur up to room temperature. If antiglobulin sera contain potent anticomplement, positive reactions may occur with RBCs from refrigerated clots. These positive reactions have been found to be largely a result of C4 sensitization, and can be avoided if the RBCs from anticoagulanted blood is used. For cross matching, RBCs from ACD or CPD segments can be used for direct antiglobulin tests; however, EDTA is preferable. These anticoagulants will chelate Ca^{++} and Mg^{++}, thus preventing any "in vitro" complement uptake, without interfering with the complement already bound to the RBC "in vivo".

(10) Red blood cells may be autoagglutinated, before they are washed, and this agglutination may persist through washing, leading to a false-positive reaction when antiglobulin serum is added.

Section III. RED BLOOD CELL ANTIBODIES

1-25. ALLOANTIBODIES

a. Alloantibodies are found in people who, through pregnancy, previous transfusion, or injections, have been exposed to foreign RBC antigens. Some people with no known immune stimulus may have unexpected antibodies, usually reacting at low temperatures.

b. Unexpected antibodies may be responsible for the following:

(1) A discrepancy between ABO and serum-grouping. This may be caused by any antibody reacting at room temperature with antigenic determinants, other than ABO, on the reagent RBCs.

(2) A positive antibody-screening test. Properly selected reagent RBCs should detect 95 percent or more of clinically significant antibodies.

(3) An incompatible crossmatch. The donor's RBCs contain one or more antigens, reacting with antibodies, in the serum of the patient.

(4) A transfusion reaction. A new antibody, in a recently transfused person, an anamnestic response caused by an antibody too weak to demonstrate before transfusion, or an antibody that was missed in pretransfusion testing may cause a transfusion reaction.

(5) Jaundice in a newborn. Both the serum of the mother and of the baby, and their RBCs must be studied. Unusual problems sometimes require study of the father's cells and those of other family members.

(6) A positive direct antiglobulin test. Antibodies may be identified in the serum, or in an eluate prepared from the RBCs. In some cases, antibodies may be directed against a drug, rather than RBC antigens.

(7) A positive autocontrol. Agglutination may be observed after room temperature (RT) or 37ºC incubation and indicate the presence of cold or warm autoantibodies, fatty acid-dependent antibodies, or abnormal proteins causing heavy rouleaux.

c. Antibodies, stimulated by RBC antigens, usually react in a predictable manner, depending on the specificity of the antigen. Some of the antigens stimulate the production of IgM and others of IgG antibodies. The IgM antibodies react by agglutinating the RBCs, while the IgG antibodies sensitize (coat) the RBCs that can then be agglutinated by antiglobulin serum. Some antibodies (both IgM and IgG) are able to activate complement. If the bound complement proceeds to completion of the sequence, the RBCs will be lysed. If the sequence is not completed, the cell-bound components can be detected with cell-bound polyspecific or monospecific (C3 or C4) antiglobulin serum (see Lesson 3, Section II).

d. The Standards for Blood Banks and Transfusion Services (SBBT) requires the serum of all donors and recipients to be tested by methods that will demonstrate hemolyzing, agglutinating, and coating antibodies.

e. The detection of antibodies in recipients is more important, than detection in donors. Mollison says, “There does not seem to be a single record in the literature of a hemolytic reaction due to destruction of the recipient’s red cells by an incompatible antibody other than anti-A and anti-B in the donor’s plasma.” He also feels that an antibody in a recipient that does not react above 30ºC has little clinical importance.

1-26. PROCEDURES

To detect antibodies with varying serologic characteristics, several different temperatures, and techniques must be used. Records should include the strength of the positive reactions and the temperature, suspending mediums, and so forth, at which reactions were observed. This information, gleaned from the antibody-screening test results or from incompatible cross matches, is useful in planning the best procedures for definitive identification and may offer some clues as to probable specificity.

a. **Saline (May be Used at Several Temperatures.)**

(1) Steps.

(a) STEP 1. Place 2 to 3 drops of serum in carefully labeled tubes.

(b) STEP 2. Add 1 drop of 2 to 5 percent suspension of reagent RBCs to each tube.

(c) STEP 3. Mix, centrifuge, and observe for hemolysis and agglutination; record the results.

(d) STEP 4. Optional. Incubate for 15 to 30 minutes at the desired temperature.

(e) STEP 5. Optional. Centrifuge; observe for presence of hemolysis and agglutination.

(f) STEP 6. Record results.

(2) Cold incubation should be carried out by using a wet ice bath rather than putting a rack of tubes in the refrigerator. If the refrigerator is used, the rack should be placed in a pan of water. There is consistently better heat transfer in a wet bath (either hot or cold). Some technologists prefer to incubate at room temperature (RT), then 15ºC to 18ºC, and then to 4ºC. There are fewer "nonspecific" results at 18ºC. Water baths can be filled with ice and water for temperature adjustment.

I (3) Incubation of a separate tube at 37ºC, rather than using the one Incubated at RT, will reduce the chances of cold agglutinins binding complement during RT incubation, and then reacting with the anticomplement components of the multispecific antiglobulin serum.

b. **High Protein.**

(1) Steps

(a) STEP 1. Add 22 to 30 percent bovine albumin, according to the manufacturer's directions, to saline tubes (if tubes were not used for 4ºC incubation).

(b) STEP 2. Centrifuge; observe for presence of agglutination or hemolysis.

(c) STEP 3. Incubate at 37ºC for 15 to 60 minutes.

(d) STEP 4. Centrifuge; observe for presence of agglutination or hemolysis.

(e) STEP 5. Record the results.

(2) Other potentiating mediums available should be used as directed by the manufacturer.

(3) The addition of albumin is considered to enhance agglutination. It also enhances the formation of rouleaux, difficult to distinguish from agglutination without microscopic observation. Cases have been reported of the presence of an antibody directed against the sodium caprylate used in the manufacture of bovine albumin. Albumin suppresses hemolysis.

c. **Antlglobulin Test.** See Lesson 1, Section ll for a complete discussion of the antiglobulin test.

NOTE: Albumin tests incubated at 37ºC.

(1) STEP 1. Fill the tube with saline, centrifuge, decant the saline, resuspend the cells, and repeat 3 or 4 times.

(2) STEP 2. Following the centrifugation for the last wash, decant all the saline and flick the last drops off the mouth of the tube. Resuspend the cell button.

(3) STEP 3. Add the amount of antiglobulin serum recommended by the manufacturer, and mix gently.

(4) STEP 4. Centrifuge, gently resuspend cells, and examine for agglutination or hemolysis.

(5) STEP 5. Record the results.

(6) STEP 6. Add one drop of known lgG-sensitized cells to all negative tests. Centrifuge; read for agglutination.

NOTE: If active antiglobulin serum is present, the sensitized cells will be agglutinated. If known sensitized cells are not agglutinated, the negative antiglobulin test is not valid and the entire procedure must be repeated.

d. **Enzyme Tests.**

(1) Enzyme methods are important additions to antibody detection and identification because they enhance the reactions of some blood-group antibodies that might otherwise be undetected, notably weak Rh and Kidd anti-bodies. On the other hand, enzymes may damage the antigenic determinants of some blood-group systems, for example, M, N Fy^a, and Fy^b; therefore, antibodies directed against these antigens may not be detected. Enzymes may be employed in a simple one-stage technique, where the enzyme is mixed directly with the test cells and the serum to be tested, or in a two-stage technique, where the test cells are pretreated before use. The one-stage procedures are used for detection, identification, and crossmatching, while the two-stage is best suited to detection and identification. Enzyme tests can be incubated at room temperature and/or 37ºC. After incubation at 37ºC, with some procedures, the cells may be washed and antiglobulin serum added. An autocontrol should always be included.

(2) It must be stressed that enzymes considerably enhance the reactions of cold autoagglutinins; therefore, many normal serums will react with enzyme-pretreated cells at room temperature, and in some instances demonstrate carry-over reactivity at 37ºC because of the presence of cold auto-agglutinins in these serums. On these occasions, it is helpful to warm the enzyme-pretreated cells and the serum under test separately to 37ºC, before mixing. This technique prevents the cold autoagglutinins from reacting with the cells below 37ºC.

(3) It should also be noted that if enzyme-pretreated cells are used with antiglobulin serum, the AHG must be shown to be free of unwanted antibody activity (that is, anti-A, -B, -H, antispecies) that might demonstrate increased reactivity with the enzyme-pretreated cells.

1-27. READING AND INTERPRETATION OF REACTIONS

a. Immediately after centrifugation, observe for hemolysis. The appearance of free hemoglobin not present in the original sample must be interpreted as a positive reaction. A white light and a white background is optimal for the observation of hemolysis.

b. The manner in which the RBCs are dislodged from the bottom of the tube is critical. The tube must be shaken gently until all cells are dislodged. This is most easily accomplished by holding the tube at a sharp angle so that the fluid “cuts” across the cell button in a manner that permits the fluid to do most of the work in resuspending the cells. When cells no longer adhere to the tube, it should be tilted back and forth gently, until an even suspension of cells (or agglutinates) is observed. Overshaking can break up fragile agglutinates, resulting in a negative reaction, while inadequate mixing may counterfeit agglutination.

c. For maximum reproducibility, observations should be made with a consistent light source. A microscope is sometimes useful in distinguishing rouleaux from true agglutination, but it is not required.

d. A scheme of grading the strength of agglutination reaction in common use is given in the following chart.

4+	one solid aggregate.
3+	several large aggregates.
2+	medium-sized aggregates, clear background.
1+	small aggregates, turbid reddish background.
W+	tiny aggregates, turbid reddish background.
0	negative, smooth suspension, no aggregates.
H	hemolysis. (This must be interpreted as a positive reaction, and may be graded as complete, or partial. When both hemolysis and agglutination are observed, this should be recorded.

1-28. REAGENT RED BLOOD CELLS

a. **Background.**

(1) Reagent RBCs may be selected from local donors or purchased commercially. The blood from selected donors is suspended in an anticoagulant-preservative solution that usually contains sodium citrate, citric acid, and dextrose, plus various other nutrients or antibiotics. The formulas used by the reagent suppliers are proprietary. One of the first formulas used for the preservation of reagent RBCs, Modified Alsever's solution, is still widely used for noncommercial collections.

(2) Most of the commercial cells are marketed, so they may be used without further preparation; however, because of the interference of a number of the common additives, washing the cells in amounts sufficient for a day's work, is suggested.

b. **For Antibody Detection.**

(1) The reagent RBCs used for antibody detection are selected to contain as many common antigens as possible. The cells are from group O donors and have been tested for the common antigenic determinants of the Rh, MNSs, Lewis, P, Kell, Duffy, Kidd, and Lutheran blood-group systems.

(2) When the cells from two or more donors are pooled, no less than 50 percent of the cells should have each of the antigenic determinants needed. Weak antibodies may not be detected when RBCs are pooled. Pooling reagent RBCs is not recommended for screening the serum of patients, but may be used when screening the serum of donors. It is likely that low-incidence antigens such as Lu^a, C^w, Kp^a, and Wr^a will not be found on detection cells. Therefore, antibodies with these specificities will be detected only when a panel must be run because of another antibody present in the serum, if a cross match is incompatible with supposedly compatible cells, or if a newborn is born with jaundice or develops jaundice soon after delivery.

c. **For Antibody Identification.**

(1) Panels of RBCs are composed of selected group O RBCs from several people tested for as many as possible of the common antigenic determinants. A panel should include some RBCs that possess--and some that lack -- these determinants, in such a manner that a distinct pattern of reaction is available for single antibodies to all or most of the antigens represented on the panel.

(2) Commercial panels will vary and contain from 8 to 10 different samples. Some include special cells either lacking a high-incidence antigen or having a Low-incidence antigen. Some provide a sample of cord cells.

(3) Another source of interesting RBCs for testing is the donor or patient found to lack either combinations of common antigens, or a high-incidence antigen, or to have a low-incidence antigen.

1-29. SERUM

a. Complement is important to the reaction of some antibodies. Serum of normal people stored at 4ºC for up to two weeks will have sufficient complement activity. It is hard to generalize about patients whose proteins may be diminished. Seventy-two hours is usually the maximum time suggested for optimal activity. Serum rather than plasma must be used because Ca^{++} and Mg^{++} ions are necessary to the complement-binding sequence.

b. Because immunologic cell lysis is an important indication presence of some antibodies, the serum should be free of hemolysis. Because this is not always possible, tests that show any degree of hemolysis should be compared with the color of the original serum.

c. Cell-free serum can be kept in the refrigerator or frozen for longer storage. Serum stored in the refrigerator on the clot will show increased hemolysis as the storage period extends. Specimens, that are to be retained for more than a few days, should have the serum removed, and the two tubes should be stopped, or covered with plastic film to minimize evaporation and contamination.

d. Serum frozen for use at a later date is best distributed into several aliquots to avoid repeated thawing and freezing. This can denature the protein. When using frozen serum, complete thawing and thorough mixing (including the volume in the dropper) are required. When serum thaws undisturbed, the protein is at the bottom and water is at the top.

1-30. CONTROLS

a. **Autologous.** Each procedure used for antibody detection or identification should include a tube containing the serum and washed cells of the person being tested. These autologous controls can then be compared to the tubes containing the reagent RBCs.

NOTE: The patient's serum is tested against his own red cells just as if they were reagent RBCs.

b. **Reagent Red Blood Cells.** Because the serum of all patients and donors must be tested for the presence of unexpected antibodies, reagent RBCs should be observed for possible deterioration. When serum or cells under test are subjected to physical or chemical alteration (as in enzyme techniques or two-mercaptoethanol treatment), reagents of known behavior must be included as controls.

c. **Control Red Blood Cells.** When unknown cells are tested with known antiserums, both positive and negative control cells should be included. The positive control should have weak expression of the antigen to be certain the serum detects weak as well as strong antigens. For example, the positive control for anti-Jk^a serum should be heterozygous Jk (a+b+) rather than homozygous Jk (a+b-) to be certain the antibody detects a single dose of the antigen because the antigen properties of the cells being tested are unknown.

1-31. ANTIBODY DETECTION

a. A routine procedure for antibody detection should be developed and be available in writing. Each member of the staff should know, and follow the directions as written. Example: Antibody detection.

(1) STEP 1. Label tubes appropriately for the screening cells and an autocontrol, (number of tubes will vary depending on type of screening cell used).

(2) STEP 2. Use the techniques described in paragraph 1-25.

(a) Saline technique. (Incubated at room temperature (RT) for 30 minutes, optional.)

(b) Albumin technique incubated at 37ºC for 30 minutes.

(c) Convert to AGT.

(d) If "(a)" is not used, an enzyme method may be valuable.

b. Various combinations may be used as long as the requirement of detecting hemolyzing, agglutinating, and coating antibodies is ensured.

c. The detection tests will indicate the methods to use for identification of the antibody. All methods showing positive results in the detection tests should be used in the identification tests. Each method could be detecting a different specificity rather than one specificity reacting by several methods.

1-32. ANTIBODY IDENTIFICATION

a. Determine the specificity of an antibody, both positive and negative reactions with the panel cells should be found. As noted in para 1-27c, a good panel should do this for most single and many multiple antibodies.

b. Preliminary testing includes ABO grouping, including the use of anti-A_1, anti-A, B, cells and A_2 cells (if indicated), Rh phenotyping, and a direct antiglobulin test. In addition, the following may be helpful: results of previous testing; history of transfusion or pregnancy; patient's diagnosis; drug therapy (including Rh immune globulin); and results of tests for whatever RBC antigens may appear to be pertinent. Preferably, these tests should be done on a pretransfusion sample; otherwise, interpretation of results is very difficult.

c. Especially useful from previous test results are such data as the effect of temperature, suspending medium, or enzyme treatment; the frequency of positive reactions with cells of random donors; the strength of positive reactions; the presence of hemolysls; and whether the antibodies appear to detect dosage.

1-33. USE OF RED BLOOD CELL PANELS

a. Each panel should be provided with a worksheet. The antigens for which the cells have been tested are listed across the top of the sheet. After the results of the reactions of the serum being tested have been recorded, the first cell showing no reactions is considered. The antigens present on the nonreactive cell are crossed out on the list at the top of the worksheet. Each nonreactive cell is interpreted in this manner. When all nonreactive cells have been considered, those antigens remaining on the list are circled.

b. If one antibody is present, all cells containing that antigen should be positive, and all cells lacking the antigen should be negative. If multiple antibodies are present in the serum, the addition of selected cells may be necessary.

c. With the exception of very rare antigens, identification should include tests with a minimum of three cells containing the antigen, and three cells lacking the antigen. The serum should react as expected with the six cells. Six cells reacting three negative and three positive provides a probability of 1/20 that the specificity is correct, with only one chance in 20 that the reactions could have occurred by chance. Probability of less than 1/20 allows for too much chance of random association. Table 1-3 gives probabilities of various combinations.

Number of Cell Samples	Reactions*		
	Positive	Negative	P
6	4	2	1/15
6	3	3	1/20
7	5	2	1/21
7	4	3	1/35
8	7	1	1/8
8	6	2	1/28
8	5	3	1/56
8	4	4	1/70
9	8	1	1/9
9	7	2	1/36
9	6	3	1/84
9	5	4	1/123
10	9	1	1/10
10	8	2	1/45
10	7	3	1/120
10	6	4	1/210
10	5	5	1/252
* No discrepancies.			

Table 1-3. Probability of identity with panels of various sizes.

d. The cells of the sample should be tested with the proper reagent serum to show they lack the incriminated antigen.

(1) Example: Antibody detection. Table 1-4.

Patient's Group: O rh (dce)													
	Antigenic Determinants									Results			
										Saline			En-
Panel cell	Rh_O D	rh' C	rh" E	hr' c	hr" e	K	k	Fy^a	Fy^b	37ºC	HP	AGT	Enzyme
1.	+	0	0	+	+	0	+	0	0	0	+	3+	3+
2.	0	0	0	+	+	+	+	0	+	0	0	0	0
3.	0	+	0	+	+	0	+	+	0	0	0	0	0
4.	+	+	0	0	+	0	+	+	+	0	0	3+	3+
5.	+	0	+	+	0	0	+	+	0	+W	2+	4+	4+
6.	0	0	+	+	+	0	+	0	+	0	0	0	0
Autologous cells	0	0	0	+	+	0	+	0	+	0	0	0	0
0 = no agglutination; HP = high protein; AGT = antiglobulin test													

Table 1-4. Example of a single antibody identified with a simplified cell panel.

(a) STEP 1. Cross out all antigenic determinants present in the first RBC sample that did not react with the serum. In this example, number two.

D C E c e K k Fy^a Fy^b

(b) STEP 2. Repeat with the next negative sample. In this example, number three thus eliminating rh' (C) and Fy^a.

D C E Fy^a

(c) STEP 3. Repeat with the next negative sample, in this example, number six, thus eliminating rh" (E).

D E

(d) STEP 4. Consider only the antigenic determinant that has not been crossed out, D.

1 Do all the cells reacting possess this determinant? Yes.

2 Do all cells possessing it react? Yes.

3 Does the patient lack it? Yes.

(e) STEP 5. Antibody identification: Anti-Rh_o (D).

(2) Example 2: Mixture of antibodies. Table 1-5.

Patient's Group: A_1 Rh_O (Dccee)

	Antigenic Determinants												Results			
	Rh_O	rh'	rh"	hr'	hr"								Saline			En-
Panel cell	D	C	E	c	e	M	N	K	k	Le^a	Le^b	RT	37ºC	HP	AGT	zyme
1.	+	0	0	+	+	+	+	0	+	0	0	0	0	0	0	0
2.	0	0	0	+	+	+	+	0	+	+	0	+	SH	2+	2+	H
3.	0	+	0	+	+	0	+	0	+	0	+	0	0	0	0	0
4.	+	+	0	0	+	+	+	+	0	0	0	0	0	0	0	0
5.	+	0	+	+	0	+	0	0	+	0	+	0	0	3+	4+	4+
6.	0	0	+	+	+	0	+	0	+	+	0	+	SH	3+	4+	H
Autologous cells	+	0	0	+	+	+	+	0	+	0	0	0	0	0	0	0

RT = room temperature; HP = high protein; AGT = = antiglobulin test; 0 = no agglutination; SH = slight hemolysis; H = hemolysis

Table 1-5. Example of multiple antibodies identified with a simplified cell panel.

Preliminary detection tests:

Saline	4ºC	RT	37ºC
0		+	Slight Hemolysis
High Protein			2+
AGT			3+

Enzyme-partial hemolysis. The cells not hemolyzed are agglutinated.

(a) STEP 1. Select the first RBC sample that did not react with the serum, in this example, number one. Cross Out all the antigenic determinants present in this sample.

D C E c e M N K K Le^a Le^b

(b) STEP 2. Repeat with the next negative sample (number three), thus eliminating (C) and Le.

C E K Le^a Le^b

(c) STEP 3. Repeat with the next negative sample (number four), thus eliminating K.

E K Le^a

(d) STEP 4. Consider only the determinants that have not been crosses out.

E Le^a

Do all panel cells that react possess these?

1 Sample number two is rh" (E)-negative but Le (a+).

2 Sample number five is rh" (E)-positive, Le (a-).

3 Sample number six is rh"(E)-positive, Le (a+).

(e) STEP 5. Interpretation: The RBCs that react are not all rh"(E)-positive nor are they all Le (a+). There is a difference in the serologic behavior (numbers 2 and 6 are hemolyzed, number 5 is not). These two observations suggest a mixture of antibodies.

1 Hemolysis of samples two and six indicate a complement-binding antibody. Antibodies of the Rh system do not hemolyze RBCs "in vitro". Therefore, the hemolysis is probably a result of the anti-Le^a.

2 Both RBC samples five and six are rh" (E)-positive. The optimum activity with high-protein, antiglobulin, and enzyme techniques is expected with antibodies of the Rh system.

(f) STEP 6. Confirmation of antibody identity:

1 The RBC phenotype of the subject is found to be Rh_o- (Dccee), Le (a-b-).

2 The serum is tested with RBCs from selected donors:

a The rh" (E)-negative, Le (a-) cells do not react.

b The rh" (E)-positive, Le (a-) cells react.

c The rh" (E)-negative, Le (a+) cells react.

3 In this example, the serum could be absorbed with rh" (E)-positive, Le (a-b-) RBCs, and the anti-rh"(E) eluted from them. The absorbed serum should still contain anti-Le^a. Absorption and elution of Lewis antibodies is not generally satisfactory. The activity of the anti- Le^a may be inhibited by using saliva or serum from a Lewis positive, preferably Le (a+) person. Although Le(a+) people are ABH nonsecretors, they have Lewis substance in their body fluids.

e. The serologic behavior of antibodies of a single specificity may vary from one serum to another. Table 1-6 lists some examples of these. Serum containing more than one antibody might give complex results; absorption, elution, and testing against specially selected cells may be necessary for final identification.

Frequency of Positive Reactions with Random Blood Samples (percent)						
Technique	Temperature (ºC)	0-10	10-50	50-75	75-90	90-100
Saline and/or albumin	4-22	Lu^a i	Le^a	Le^b	MN P_1	I
	22-37	$rh^w(C^w)$ Lu^a	rh"(E) Le^a S	rh'(C) Le^b	$Rh_O(D)$ hr'(c) s	hr"(e) k U Vel
Saline and/or albumin plus antiglobulin	37	$rh^w(C^w)$ K Kp^a Lu^a Di^a Yt^b	rh"(E) Le^a S Js^a	rh'(C) Le^b Fy^a Jk^b Xg^a Do^a	$Rh_O(D)$ hr'(c) Fy^b s Jk^a Au	hr"(e) k U Kp^bGe^a Lu^bYt^a Vel Js^b Di^b
Enzyme	37	$rh^w(C^w)$	rh"(E) Le^a	rh'(C) Le^b	$Rh_O(D)$ hr'(c)	hr"(e)
Enzyme plus antiglobulin	37	$rh^w(C^w)$	rh"(E) Le^a	rh'(C) Le^b JK^b Do^a	$Rh_O(D)$ hr'(c) JK^a	hr"(e)
Hemolysis Saline	37		Le^a JK^b	Le^b	JK^a	Vel
Hemolysis Enzyme	37		Le^a	Le^b JK^b	JK^a P_1	
All techniques & temperatures			Le^a	Le^b		PP_1P^k

Table 1-6. Antigenic frequency and serologic reactivity.

1-34. ANTIGENIC FREQUENCY

a. It may be possible to get some idea of antibody specificity early in an investigation, thus saving time when the need for blood is urgent.

b. A request for five units of blood is made in an emergency. The blood specimen from the patient is tested for ABO, Rh, and antibodies. The antibody-detection test shows two plus agglutination at RT, no agglutination at 37ºC, and is negative by AGT. While this test is incubating, five units of the appropriate ABO and Rh group are cross matched. Of the five units selected; four are agglutinated after centrifugation at room temperature (RT) and one is not. The unagglutinated specimen is continued through the cross match technique. At the same time, the patient's serum is tested with the RBC panel.

c. Table 1-6 includes three antibodies that react at RT with a frequency of 75 to 90 percent: anti-P, anti-M, and anti-N. The RBCs of the patient are tested with reagent antiserums of the three specificities. Within a short time, the information is available that the patient is P_1, NN. The RBCs that are MM or MN are agglutinated while those that are NN are not.

d. The four incompatible units are MM or MN and the one compatible is NN. The antibody is probably anti-M. Additional units can be tested with anti-M, and those that are negative can be crossmatched. Meanwhile, one unit is available.

1-35. INTERPRETATION OF COMMON PANEL RESULTS

a. Some cells are positive, some negative; autocontrol negative.

(1) One or more specific antibodies are present. With single antibodies, the pattern of reaction is often evident; however, the possibility of additional antibodies should not be ignored. This is the reason for the crossing out of antigens described in paragraph 1-32d, step 2.

(2) When several antibodies coexist, the reaction temperatures and reaction strengths usually offer clues as to specificities.

(a) At RT, 18ºC or 4ºC. The antibodies most often identified by a cold panel are Lewis, I, M, N, and P_1.

(b) At 37ºC. Antibodies most often identified after incubation at 37ºC, in saline or albumin, are the Rh antibodies, anti-KeII, -S and -s.

(c) After AGT. After addition of antiglobulin serum, almost all warm antibodies can be detected: anti-Kell, -Duffy, -Kidd, and so forth

(d) Hemolysis. Antibodies most often hemolyzing saline-suspended cells are anti Le^a and Kidd antibodies.

(e) Enzymes. Anti-Fy^a, -FY^b, -M, -N, -S may not be detected when enzymes are used. Rh antibody reactions are enhanced by the enzyme procedures in common use. In fact, these procedures were developed originally to detect Rh antibodies. Conversion of the test to AGT will aid in identifying weak Kidd antibodies. In addition to the antibodies listed in d, anti-P_1 may also be hemolytic in an enzyme system.

b. All panel cells are equally positive; autocontrol negative.

(1) An antibody against a high-frequency antigen or a mixture of antibodies may be present.

(2) Testing against specially selected cells is necessary for identification.
.

(a) At RT. 18ºC or 4ºC. Antibodies that fit this pattern include anti-Sd^a, anti-I in an I-negative person, anti-H or -HI in individuals of group A_1, A_1B, or (infrequently) B. Serums from Bombay or p people also react in this way.

(b) At 3º7C. Antibodies against high-incidence antigens that may react are anti~Lu^b and Vel.

(c) After AGT. Almost all antibodies for high-incidence antigens will react by the antiglobulin test. The more common are: anti-k, Kp^b, -U, -Yt^a, -Vel, -Lan, -Js^b.

(d) Hemolysis. The antibodies for high-incidence antigens that cause hemolysis “In vitro” are anti-Vel and -PP_1P^k.

c. All panel cells are autocontrol positive at RT; stronger at 4ºC: weaker at 37ºC; antiglobulin phase variable, usually weak. A cold antibody, usually anti-I, with autologous activity may be present. Rouleaux formation and fatty-acid-dependent antibodies may give a comparable picture. Since cold autoantibodies may mask the simultaneous presence of alloantibodies, the serum should be autoabsorbed, and the antibody-screening and identification procedure repeated on the absorbed serum.

d. Cells on panel are negative or variably positive; autocontrol positive in antiglobulin phase. The subject’s cells may have a positive DAT at the same time that an alloantibody is present in the serum. In autoimmune hemolytic anemia, all antibodies may be bound to the cell so that none will be detectable in the serum (see Lesson 2, Section I).

e. All panel cells and autocontrol are positive only in AGT. The subject may have a warm autoimmune antibody (see Lesson 2, Section I).

f. All cells in the panel negative; autocontrol negative; many cross-matches incompatible. This may be found when the pre-panel work has not been properly done. Anti-A_1 occurring in a small number of A_2 and A_2B individuals, produces incompatibility in approximately 80 percent of crossmatches involving random group A blood (that is, all A_1 or A_1B bloods), but does not react at all with the group O cells on the panel.

g. All cells in the panel negative; autocontrol negative; rare cross- match positive; or cells of infant are incompatible with mother. The serum may contain an antibody for a low-incidence or familial antibody. Many of the low-incidence antibodies were first recognized when a newborn, expected to be normal, was born with, or developed hemolytic disease of the newborn during the neonatal period.

1-36. ANTIBODIES NOT EASILY IDENTIFIED

When the results with the RBC panels do not permit clear identification, the following should be considered.

a. There are occasions when a panel may have to be other than group O. To prove a presence of an anti-A_1 in a group A_2 or A_2B, the cells have to be divided between A_1 and A_2. If, in addition to anti-H, another antibody is present in the serum of a group A_1-individual, the panel cells would all have to be A_1 that have been tested for the other common antigens.

b. More than one antibody may be present. Look at the reactions of the antibody with the cells on the panel. Are there differences among the reacting cells in temperature, suspending medium, or strength of reaction? Do some cells hemolyze? Are some reactions enhanced or destroyed with enzymes? This may give an indication of the identity of one or more of the antibodies. Absorption and elution studies may be used to separate the antibodies. Type the subject's cells to determine their antigenic composition. Except in autoimmune diseases, antibodies are not present in the subject's serum if the antigens are on the RBCs. If the cells have a positive DAT, serums reacting by AGT cannot be used in the routine manner.

CAUTION: Testing should be performed on <u>pretransfusion</u> cells. Circulating donor cells contribute an assortment of foreign antigens that may be misleading.

c. The antibody may be reacting in an unexpected manner. One would not expect anti-M or anti-P_1 to react only by AGT or anti-s to react only at RT, but it could happen.

d. Weak antibodies may be reacting only with RBCs from donors homozygous for the antigen concerned or only with those cells possessing the strongest antigens.

e. The antibody may be reacting with an antigen for which the RBCs have not been tested. For example, the antibody may be reacting with 65 percent of random donors and with several cells of the panel, but without a specific pattern. If the pattern has not been typed for Dombrock (Do^a), one might suspect this antibody.

f. The antibody may be directed against a low-frequency antigen not present on the panel. This is usually encountered when the cross match or antibody-screening test is positive, and the panel is negative. The antigen is on the screening cells of the donor's cells, but not on any other cells tested. The commercial distributors of the cells frequently have Information about the cells that are not on the protocols distributed. They will provide this information on request. Antibodies to low -incidence antigens may cause hemolytic disease of the newborn, in which case, maternal antibody reacts only with the RBCs of the infant, the father, and possibly some members of his family, but the antibody-screening test and panel results are negative. These cases can sometimes be identified by testing the father's cells with known antibodies for low-incidence antigens and testing the unknown antibody against cells known to possess low-incidence antigens.

g. When all cells are agglutinated, the antibody may be reacting with a high-incidence antigen. Test the antibody with known cells that lack high-incidence antigens, and test the patient's pretransfusion cells with known antibodies for high-incidence antigens. If a patient lacks a high-incidence antigen and has an antibody that reacts with all the cells tested, chances are very good that the antibody is directed against that antigen. It is wise to use several cells that are negative for their high-incidence antigen in order to demonstrate the presence of any other antibodies. Persons, who have developed one antibody, and have proved their antibody production ability should be suspected of having more than one antibody until proved otherwise. Compatible donors suspected can sometimes be found among the siblings of the patient, and usually from the AABB Rare Donor File.

h. When all the panel cells react by forming weak, fragile agglutinates, the antibody may have one of the high-titer, low-acidity specificities. These are a group of antibodies that may show titers into the hundreds; but not more than two plus reactIons in the strongest dilution. Two of these, anti-Chido (Ch^a) and anti-Rodgers (Rg^a), can be inhibited by plasma from Ch (a+) and Rg (a+) persons, but not by other secretions. Many of these antibodies have been shown to be related to antigens on white cells. Some distinguishing features are shown in Table 1-7.

	Antibodies								
Blood Cell Reactions	Ch[a]	Rg[a]	Sd[a]	Yk[a]	Cs[a]	Kn[a]	McC[a]	Ge[a]	Ve[a]
Positive AGT of variable strength with RBCs of more than 90 percent donors	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Weaker against cord RBCs	Yes	Yes	Yes	No	No	No	No	No	Yes
Weak against enzyme-treated RBCs	Yes	Yes		No* Yes+		No		No	
Specifically inhibited by									
-plasma	Yes	Yes	No	No	No	No	No	No	No
-saliva	No	No	Yes	No	No	No	No	No	No
percent frequency of donors lacking antigen									
White	2	3	9	8	2.5	0.05	1	0.02	0.02
Black				1.8	3				

* Papin-treated cells.
\+ Ficin-treated cells.
From Crookston.

Table 1-7. Distinguished features of some antibodies that react with almost all random donors.

1-37. USEFUL ADDITIONAL NOTES

a. Rouleaux can be identified by microscopic examination of autologous cells in their serum. The cells adhere to one another on their flat surfaces, giving the classic "stack of coins" appearance. These groups tend to roll and tumble together, but disperse with the addition of saline, while true agglutination will not. True agglutinates are opaque, inflexible, and have an irregular outline. In assessing rouleaux formation, knowledge of the patient's clinical diagnosis and of the protein content and proportions in the serum is helpful. Problems resulting from rouleaux can be encountered at any step of the procedure, but usually, they occur in high-protein methods.

b. Some serums contain antibodies that will react with sodium caprylate used as a stabilizer in the production of some albumlns. Serum containing caprylate antibody agglutinates containing caprylate antibody agglutinates albumin-suspended cells, including autologous cells. Despite the autoagglutination, the direct antiglobulin test is negative. The phenomenon is caused by a fatty-acid-dependent antibody apparently specific for sodium caprylate. Such serums do not agglutinate identical cells when suspended in saline or in albumin prepared without caprylate. Usually all cells and the autologous control are affected equally.

c. Previously immunized patients who have recently been transfused can very rapidly produce antibody against one or several antigens on the transfused cells. The antibody then reacts with the circulating antigen-positive cells and, if activity is sufficiently strong, may also be found in the serum. The posttransfusion direct antiglobulin test (DAT) will be positive with a mixed-field appearance, because only the transfused cells contain the antigen.

d. Coexistence of cold and warm antibodies may be suspected when the antibody-screening test demonstrates activity at RT, 37ºC, and AGT. This can be resolved by preparing two separate panels: one incubated-from RT to 18ºC, or 4ºC, the other at 37ºC. lgM antibodies can be neutralized by the use of 2-mercaptoethanol or dithiothreitol. This is used when the mixture of antibodies has the possibility of being lgM and lgG.

e. Reaction strength should be graded. Variations in the strength of reactions may indicate the following:

(1) Dosage. The antibody may be reacting more strongly with cells that are homozygous for the antigenic determinant than with the heterozygous cells. For example, anti-M may react more strongly with MM cells than with MN. Some antibodies that may demonstrate a dosage effect are anti-M, -N, -JK^a, -JK^b, some Duffy antibodies, and some Rh antibodies.

(2) Variation in the strength of the antigen. Some antigens that do not show dosage in terms of zygosity may vary in strength on cells from different individuals. The antigens most notable for this are P_1, Sd^a, Le^a, Le^b, and I. Sometimes diminished antigenic strength can be related to disease, or to the length of time that the cell suspension has been stored.

1-38. COLD ANTIBODIES

a. **Background.** These antibodies react best at room temperature (RT) and below, while reactions after 37ºC incubation and with AGT are negative or weakly positive. Some cold agglutinating antibodies, notably anti-I, anti-IH, and anti-H, bind complement following the antigen-antibody interaction at low temperatures; subsequent 37ºC incubation may cause the antibody to elute from the cell, but the attached complement remains, and produces a positive reaction when antiglobulin serum is added. An antibody displaying these characteristics in the screening test or in crossmatches should be tested at 18ºC and/or 4ºC with specially selected cells, such as cord cells.

b. **Cold Autoabsorption to Remove Cold-Reactive Autoantibody.**

(1) When a strong, cold, autoantibody is present, meaningful antibody-screening and compatibility studies cannot be done until the autoantibody is removed. This can be done with the patient's untreated or enzyme-treated cells. Enzyme pretreatment results in faster antibody removal, and is more effective with very high-titered autoantibodies. Untreated autoabsorption is usually satisfactory for removing low-level activity.

(2) Some alloantibody may also be removed by the Matuhasi-Ogata phenomenon, or by transfused cells in the circulation. In recently transfused patients, when autoabsorption might result in removing alloantibody activity, the antibody-screening test and compatibility tests should be done at 37ºC, using serum from a blood sample clotted at 37ºC and kept at that temperature until the serum is removed. This permits detection of antibodies active at 37ºC, and by AGT, but not cold-reactive alloantibodies.

(a) STEP 1. Obtain a blood specimen from the patient. At the bedside draw two specimens of blood, one in a tube immediately placed in ice, the other in a tube containing anticoagulant and incubate it at once at 37ºC. To do this, bring a heated block, water bath, or vacuum jug of 37ºC water to the patient's bedside. The tube may also be transported in an inside pocket.

(b) STEP 2. Allow the first (iced) specimen to clot in the refrigerator. Separate the serum by centrifuging in ice-filled cups or a refrigerated centrifuge. Immediately remove the serum from the clot.

(c) STEP 3. Remove plasma from the 37ºC incubated cells. Wash the cells 3 times, in a large volume of warm (37ºC) saline, using warmed centrifuge cups.

(d) STEP 4. Prepare a small volume of 2 percent to 5 percent suspension of the RBCs in saline, using the washed, packed, RBCs from step 3. Use these cells for ABO and other testing.

(e) STEP 5. Test the RBCs from step 4 with the serum from step 2, at 4°C. If cold agglutinin was removed in the initial incubation, tests will be negative.

(f) STEP 6. If antibody remains, add 1 volume of serum to 1 volume of patient's washed, packed, cells from step 3, and place in an ice bath for 30 to 60 minutes. Shake frequently for maximum absorption.

(g) STEP 7. After cold centrifugation, remove the supernatant serum as quickly as possible, and test with autologous RBCs from step 4. Test at 4ºC as in step 5. The procedure should be repeated if cold antibody remains.

c. **Absorption with Enzyme-Treated Cells.**

(1) STEP 1. With one volume of washed, packed, autologous RBCs, add one volume of enzyme solution. Any enzyme may be used (see the Special Methods section).

(2) STEP 2. Mix and incubate at 37ºC for 15 minutes.

(3) STEP 3. Wash cells several times to remove enzyme solution. Be sure to mix the cells well with each new volume of saline. This is best done by adding half a tube of saline, mixing the cells by inversion to remove them all from the bottom, then filling the tube with saline.

(4) STEP 4. To one volume of washed, packed, enzyme-treated cells, add one volume of patient's serum.

(5) STEP 5. Mix and incubate in ice bath for 15 to 60 minutes; mix frequently for maximum absorption. In some cases when the antibody is very potent, an even shorter incubation will result in almost complete agglutination of the cells.

(6) STEP 6. Centrifuge and harvest serum, as quickly as possible.

(7) STEP 7. Test patient's absorbed serum against autologous cells to determine if cold autoagglutinin has been completely removed. If not, repeat absorption with fresh enzyme-treated cells.

d. **Identification of Cold Autoantibody.**

(1) The specificity of cold autoantibodies can be demonstrated by testing against a reagent RBC panel, or the special panels described below. Cord cells (i) should be used, and (i) adult cells, if available. For the autocontrol, cells free of the absorbed antibody should be used. (See step three in cold-absorption procedure.)

(2) If agglutination patterns are unclear, a clue to, specificity can often be achieved by testing diluted serum against cells positive and negative for the suspected antigen, usually I or one of the I complexes. In Table 1-8 the specificity is anti-I and the patient's RBCs react as strongly as other I-positive cells.

NOTE: When such titration studies are done, it should be borne in mind that any alloantibody contained in the serum will also be diluted. If the alloantibodies are of low titer, they may then be undetected. For this reason, autoabsorbed serum is preferred.

Test Red Blood Cells							
Dilution	I-pos	I-pos	I-pos	i (adult)	i (cord)	i (cord)	Auto
Neat	++++	++++	++++	+	+	+	++++
1:16	++	++	++	W+	W+	W+	++
1:32	+	+	+	0	0	0	+
1:64	W+	0	W+	0	0	0	W+
1:28	0	0	0	0	0	0	0

Table 1-8. Identification of anti-I.

e. **Special Panels.**

(1) Anti-I agglutinates all adult cells, except those from rare i-adults, but gives much weaker or negative results with cord cells containing small amounts of I antigen and much larger amounts of i antigen. Anti-i agglutinates cord cells and the rare adult i cells much more strongly than the majority of adult cells.

(2) Anti-IH agglutinates cells according to the amount of H present on the cells: group O cells have the most, then A_2, B, A_1, A_1B. Group O cord cells react somewhat less strongly with the anti-H than group adult cells.

(3) Anti-IH agglutinates only those cells possessing both H and I antigens; thus, it does not agglutinate group O cord cells.

(4) Anti-A_1 agglutinates A_1 or A_1B cells, but does not affect A_2 or O cells.

(5) The technique is the same as for the cold panel. Washed, fresh cell samples should be used in a 2 percent to 5 percent saline suspension.

(6) Table 1-9 classifies the reactions of some cold antibodies using a special panel.

	Cells					
Unknown Serum	**^1**	**^1**	**Adult O**	**Cord O**	**Auto Control**	**Interpretation**
1.	0 or W+	+++	++++	++	0 or W+	Anti-H
2.	++	++	++	0 or W+	0 to ++++	Anti-I
3.	0 or W+	++	++++	0 or W+	0 or W+	Anti-IH
4.	0 or W+	0 or W+	0 or W+	+++	0 or W+	Anti-i

Table 1-9. Identification of antibodies with a special panel of cells.

(7) Adult i cells should be used in addition to cord cells, if available. Cord cells also have weak expressions of Lewis, Sd^a, Chido, and may have very weak P_1 antigens. A serum failing to agglutinate cord cells cannot reliably be called anti-I, until these antibody specificities have been eliminated.

1-39. TITRATION

a. **Background.**

(1) Titration is a semi-quantitative means of measuring the amount of antibody in a serum. Serial dilutions (usually two-fold) of antibody are tested with a constant volume of RBCs, and the result is expressed as the reciprocal of the highest dilution, at which agglutination is observed. This is usually a macroscopic observation, but in techniques such as those used in high-titer, low-acidity testing, it is microscopic.

(2) The physical-chemical properties of the antibody, of the reaction conditions, and of the reagent cells can all influence the results. Therefore, these variables must be standardized as carefully as possible. Because of its semi-quantitative nature, titration is most useful in comparing one serum with another rather than attempting to provide absolute information. Titration is most often used to demonstrate changing amounts of antibody during pregnancy; however, it is most useful when comparing one serum against several cell samples to clarify differences between alloactive, and autoactive antibodies in a serum (for example, identifying anti-I activity or in identifying "least incompatible" donor units) when crossmatch difficulties exist.

b. **Factors Affecting Titration.**

(1) Ideally, when several serums are compared, cells from the same donor, freshly drawn and prepared, should be used for each titration. If commercially prepared reagent cells are used, the same genotype should be employed consistently.

(2) If the antibody is diluted with saline, the RBCs should be suspended in saline. If a high-protein medium is used for dilution, the RBCs should be suspended in albumin or serum.

(3) Meticulous pipetting technique is necessary for meaningful titration results. Mouth pipetting is prohibited. Semiautomatic pipettes are recommended. A clean pipette tip should be used for each dilution.

(4) Results should be read macroscopically. The prozone phenomenon may produce weaker reactions in the first one or two tubes, than in the higher dilutions, so the entire series of tubes should be evaluated, starting with the most dilute, and ending with the most concentrated sample.

(5) Optimum incubation time, temperature, and centrifugation condition should be determined in preliminary evaluation of the antibody. Once determined, these should be used consistently.

(6) If serums are to be compared, the titrations should be done at the same time. With prenatal specimens, successive samples should be stored, frozen, for comparison with subsequent specimens. Each specimen should be tested along with the immediate preceding sample.

NOTE: Only a titer change of two tubes or more is significant.

c. **Technique for Single Titration.**

(1) STEP 1. Label a row of tubes according to the serum dilution, usually 1:1 through 1:512.

(2) STEP 2. Deliver 0.1 ml of saline into the bottom of all tubes, except the first.

(3) STEP 3. Add 0.1 ml serum to tubes 1 and 2 (dilutions 1:1 and 1:2).

(4) STEP 4. With a clean pipette, mix the contents of tube 2 several times; then transfer 0.1 ml to tube 4 (1:4 dilution).

(5) STEP 5. Continue same technique through all dilutions. Remove 0.1 ml from 1:512 tube, and save for use in further dilutions if needed.

(6) STEP 6. Add 0.1 ml of saline suspension of appropriate RBCs to each tube.

(7) STEP 7. Incubate in appropriate manner, according to the antibody being tested.

(8) STEP 8. Centrifuge.

(9) STEP 9. Gently dislodge RBC button, and observe macroscopically for agglutination, starting with the 1:512 tube. Record the results. Express this end point as saline titer.

(10) STEP 10. All unagglutinated or weakly agglutinated specimens are then washed 3 or 4 times for the AGT, if this is desired. Express this end point as antiglobulin titer.

d. **Technique for Master Dilution.** When a diluted serum is to be studied for reactivity against several RBC specimens, the dilutions are kept constant by preparing a master dilution. Use larger volumes of serum and diluent, and larger tubes (if needed). The diluted serum for each tube in each titration series can now be transferred from the corresponding master tube. Even if only one cell is to be used, a large-volume dilution may be easier than the 0.1-mi technique.

e. **Scoring.**

(1) If two or more antibodies are to be compared, it may be desirable to evaluate more than titer. By grading the strength of reactions at each dilution, one achieves a better understanding of the total activity, which includes strength of reaction as well as concentration. It is essential to use the same cell suspension for all the specimens involved, and to employ rigidly standardized technique. The numerical score assigned to specific degrees of agglutination varies among laboratories, but it should be uniform for all workers in any one laboratory.

Qualitative Notation	Score	
4+	12	(Complete or partial hemolysis cannot easily be quantitated. But usually denotes a highly positive reaction.)
3+	10	
2+	8	
1+	5	
W+	2	
0	0	

(2) The following example of titration and scoring shows how results may be misleading, when evaluating the relative potency of serums. The same cell suspension is used for both serums.

Example

Mrs. K.M., 1970	1	2	4	8	16	32	64	128	Total Score
Strength of reaction	4	3	3	2	2	1	1	0	
Score	12	10	10	8	8	5	5	0	58
Mrs. K.M., 1974									
Strength of reaction	4	4	4	4	3	2	1	0	
Score	12	12	12	12	10	8	5	0	71

The two serums have titers of 64, yet the difference in score indicates that there has been an increase in antibody strength.

1-40. ABSORPTION

a. **Background.**

(1) Absorption is removing antibody from a serum by reacting it with the specific antigen, and then physically separating the antigen- antibody complexes from the serum. Intact RBCs are the usual source of antigen, but RBC stroma is occasionally used. Situations in which absorption may be useful include:

(a) Removal of cold or warm autoantibody activity to permit evaluation of coexisting alloantibody.

(b) Removal of anti-A and/or anti-B from serum containing an antibody, suitable for reagent use.

(c) Separation of mixed antibodies in serum, or eluate, as an aid to identification.

(d) Documentation that cells contain an antigen, through demonstrating their ability to remove antibody from serum.

(2) The intended purpose of absorption dictates, to some extent, the necessary techniques. When interfering autoantibodies are to be removed, several absorptions may be necessary, using a fresh aliquot of cells each time. If the goal is to separate mixed antibodies, the cells must be selected to contain only one of the antigenic determinants in question, preferably, that giving the strongest possible reaction with the antibody.

b. **Serologic Considerations.**

(1) The temperature should be optimal for the activity of the antibody to be removed. If both cold and warm antibodies are present, separation is enhanced by absorbing the cold activity at 4ºC, or the warm activity, at 37ºC.

(2) The usual volume is one part undiluted serum to one volume, washed, hard-packed, cells. For complete absorption of high-titered antibodies, diluted serum may be more efficient. With weak antibodies, the volume of undiluted serum should be greater than the volume of RBCs.

(3) Specific techniques for warm and cold absorption of autoantibodies have been given in appropriate sections.

c. **Techniques.**

(1) STEP 1. Wash selected RBCs, at least three times, with isotonic saline. The cells may be heterologous for antibody separation, and identification, or autologous, for autoantibody removal. Completely remove supernatant saline after last washing to avoid dilution of the serum. If the RBCs were in contact with serum or plasma containing cold antibodies, washing at 37° may be indicated.

(2) STEP 2. The larger the area of contact, the better the absorption. This is particularly true in prolonged absorption, when the RBCs settle. Use a large-bore tube. Stopper well and lay tube on side. For volumes of cells and serum, see b (2) above.

(3) STEP 3. Incubate the packed RBCs and serum at optimum temperature, for the reaction, for 30 to 60 minutes, agitating frequently.

(4) STEP 4. Centrifuge. If absorption is at 4ºC, use prechilled centrifuge cups, or a refrigerated centrifuge. Centrifugation at higher temperatures will result in elution of antibody from the RBCs.

(5) STEP 5. Remove the serum immediately after centrifugation. When this serum is used for additional tests, identify it as absorbed serum. If one serum is to undergo absorption with more than one cell, the absorbed serum should also be identified by the cell specificity, (for example, absorbed DccEE).

(6) STEP 6. Test the absorbed serum for complete absorption, using the method of optimum reactivity, against a freshly prepared suspension of the RBCs used for absorption. If the absorbed serum still reacts with the cells, absorption has not been complete, and should be repeated with a fresh aliquot of washed, packed cells.

(7) STEP 7. If an eluate is to be made, save the cells with the absorbed antibody.

1-41. ELUTION

a. **Background.** Elution is removal of antibody that has been absorbed onto RBC surfaces. The absorption may have been “in vivo” (autoimmune) or "in vitro.” Elution may be undertaken for a number of reasons:

(1) To demonstrate and identify the antibody of an infant’s cells in hemolytic disease of the newborn. If the mother’s serum is unavailable, the eluate may be used for crossmatching for exchange transfusion.

(2) To demonstrate and identify the antibody producing the positive antiglobulin test in acquired hemolytic anemias, or in suspected transfusion reactions.

(3) To produce small amounts of useful single-antibody preparations after a mixture of antibodies has been separated by absorption onto cells of the appropriate phenotype.

(4) To free the cells from adsorbed antibody (usually autoantibody, cold-active) so they are suitable for further testing, or for auto-absorbing the serum.

(5) To demonstrate that cells have adsorbed an antibody; and therefore, possess an antigen, despite the failure of the antibody to agglutinate the cells. (This is usually done with weak antigens of the ABO system, since antiglobulin-testing provides this kind of information in the Rh and most other systems.)

b. **Preparation of Cells.** In all elution methods, the most critical detail is the complete removal of unabsorbed antibody surrounding the RBCs. The cells must be washed at least three times; in some cases, as many as 12 times. The procedure for cell-washing is the same for all methods. To test for complete removal of unabsorbed antibodies after the third washing and centrifugation, remove all the supernatant fluid except for the 0.5 ml directly over the RBC mass. Remove this fluid and test it with RBCs known to react with the “unwanted” antibody. If the test is negative, proceed with the preparation of the eluate. The eluate usually contains hemoglobin, which does not interfere with testing. Because complement is not

present in the eluate, hemolysis of the cells used in testing is not expected. Since eluates may not be stable, it is preferable that they be tested on the day of preparation. If storage is anticipated, eluates should be prepared in AB serum or 6 percent albumin and frozen at -20°C or lower.

c. **Heat Elution.**

(1) STEP 1. Add a volume of saline, (or group AB serum or 6 percent (albumin), to each volume of packed, washed, RBCs.

(2) STEP 2. Mix well, and place in a 56ºC water bath, approximately 10 minutes, agitating frequently during incubation.

(3) STEP 3. Immediately transfer the tube to prewarmed centrifuge cups containing 56°C water.

(4) STEP 4. Centrifuge at high speed.

(5) STEP 5. Remove the hemoglobin-tinted supernatant eluate.

NOTE: If care is taken and the cells agitated gently for five minutes, it is possible to remove the antibody, and still have intact cells. The eluate is very slightly hemolyzed.

Section IV. DETERMINATION OF COMPATIBILITY

1-42. DETERMINATION OF COMPATIBILITY

a. Compatibility testing consists of a series of procedures performed by the blood bank, before transfusion, to ensure the proper selection of blood for the patient. These procedures should include the following:

(1) A review, of the blood bank records, for results of previous testing to check for the recipient's group, type, and for any unexpected RBC antibody that may have been previously identified.

(2) ABO grouping, Rh typing, and red cell antibody detection on each recipient sample, sent for compatibility testing.

(3) ABO grouping, Rh typing, and red cell antibody detection on the unit of blood. (The antibody detection test need not be repeated if it has been performed by the collecting agent.)

(4) Cross matching.

b. Careful technique and complete concentration are necessary in testing, since incorrect results can directly endanger the life of the recipient.

c. The AABB Standards and the FDA (BoB) require the testing of the donor's cells with the recipient's serum, (major crossmatch), by a method that will demonstrate agglutinating, coating, and hemolyzing antibodies, which, shall include the antiglobulin method. Antihuman globulin reagent for the antiglobulin test shall meet FDA (BoB) standards.

d. Testing donor serum with recipient RBCs (minor crossmatch) need not be included, since donor serum must be tested initially for hemolyzing, agglutinating, and coating antibodies with reagent RBCs, meeting FDA (BoB) standards.

e. The crossmatch will detect the following:

(1) Most recipient antibodies, directed against antigens on the donor RBCs.

(2) Major errors in ABO grouping, labeling, identification of donors, and recipients.

f. The crossmatch will not:

` (1) Guarantee normal donor cell survival.

(2) Prevent recipient immunization.

(3) Detect all ABO grouping errors.

(4) Detect Rh typing errors, (unless the recipient's serum contains Rh antibody from previous immunization).

(5) Detect all unexpected RBC antibodies in recipient serum. Clerical errors are more common than technical ones in a blood bank, or transfusion service. Nontechnical mistakes, such as inadequate or incorrect identification of the recipient or donor, are usually caused by not adhering to established protocols.

1-43. COLLECTION OF BLOOD FROM RECIPIENT

a. Blood request forms must include the recipient's full name, and identification number, and because blood is a drug and for other medical-legal reasons, the name of the responsible physician should appear on the requisition form. Additional information such as sex, amount of blood, or component requested may be helpful, but this is not required by the AABB Standards. Only completed forms may be accepted. Transmittals from a computer program are acceptable. Telephone requests should be confirmed in writing with a properly completed request form.

b. The recipient and the blood sample must be positively identified, when the sample for compatibility testing is drawn. One way, of positive identification, is to ask the patient to state his full name, not merely to confirm it. If the patient is unable to state his name, the wristband must be relied upon totally for information. When a wristband (or other identification attached to the person) is not available, it is necessary to confirm the identification with a professional person familiar with the patient. Bed labels should not be used in place of wristbands. The unidentified emergency patient should be given a temporary identify number (attached to his person) that can be used until positive identification is made.

c. The wristband should then be checked, and the name and identification number should be copied from the source onto the tube label, with an indelible marker. If imprinted labels are used, the information on the labels should match the wristband exactly. The date should then be added, and the identification compared to the request form. Other pertinent data may appear on the tube label, such as, initials of the person responsible for collecting the recipient sample. The tube must be labeled immediately before, or after, the blood is drawn at the bedside of the recipient. Ordinarily, the sample should not be drawn from tubing used for infusion of intravenous fluid or from the contiguous vein, but from a fresh venipuncture site. If the tubing must be used, it should be flushed with saline and the first 5 ml of blood withdrawn should be discarded.

1-44. PRETRANSFUSION TESTING OF THE RECIPIENT

The information on the request form and sample label must be compared by a qualified person before testing can begin. In case of discrepancy or doubt concerning the specimen, a new sample must be drawn.

a. **Patient Specimen.**

(1) Fresh, not inactivated serum, less than 72 hours old must be used for the crossmatch. If plasma is used, fibrin clots may form and interfere with the test and with complement activation.

(2) When a series of transfusions is to be given over a period of several days, a new sample of the recipient's blood obtained within 72 hours of the next scheduled transfusion should be used. This is essential for the detection of antibodies that may appear in the recipient's circulation in response to blood previously transfused. If more than 72 hours have elapsed since the previous transfusion, units of donor blood previously crossmatched must be recrossmatched with a new patient serum specimen before transfusion.

(3) Hemolyzed patient samples should be avoided because they may mask hemolysis of donor RBCs.

(4) A washed RBC suspension may be prepared. Red blood cells suspended in serum should not be used if the serum contains autoagglutinins, cold agglutinins, or abnormal proteins that cause rouleaux.

b. **Preliminary Testing.** The following are imperative before crossmatching.

(1) A review of blood bank records for results of previous testing.

(2) ABO grouping.

(3) Rh typing and Rh_O(D) and control. Special care must be taken in testing the recipient who may have received transfusions of an ABO group or Rh type different from his own, since transfused cells may give misleading results.

(4) The screening of recipient sample for unexpected antibodies. This may be performed at the same time as the crossmatch. Identify the antibody if the screen is positive.

1-45. SELECTION OF BLOOD

a. **Background.**

(1) The blood selected for crossmatch should be of the same ABO group and Rh type specific as that of the recipient when whole blood is being transfused; however, there are instances when it is acceptable, or even advisable, to transfuse RBCs of a different ABO group, provided that they are compatible. For example:

(a) When group-specific and type-specific blood is unavailable, as in transfusing group A blood to an AB recipient.

(b) For ABO and/or Rh hemolytic disease.

(2) It is not necessary to be concerned with subgroups of A, unless the patient has a clinically significant anti-A_1 or anti-'Hl'(O). Anti-'Hl'is also referred to by different authors as anti-0, anti-IH, and anti-H_O. Those examples of anti-O('Hl'), that are not inhibited by H substance, are occasionally active at temperatures above 30ºC, and have been known to cause rapid destruction of transfused incompatible RBCs. These patients should receive A_1 blood. Anti-A_1, which is active "in vitro" at 30ºC higher, has been shown to be capable of extensive red cell destruction. Therefore, these patients should receive A_2 blood.

(3) Matching of the Rh-Hr antigens other than Rh_O(D) is not recommended, unless the patient has a known Rh-Hr antibody, in which case, blood lacking the corresponding antigen must be selected, but it is not necessary to type donor blood for additional antigens.

(4) The expiration date of the blood should be noted, to be sure that the age of the blood is acceptable for its intended use.

b. **Choice of Blood When Group-Specific Blood Is Unavailable.** When blood of the recipient's ABO group is unavailable, transfusion with an alternate group, as shown, is acceptable, but must be administered as RBCs, or. if whole blood, must be shown to lack hemolvsins directed against cells of the patient's group.

	Alternate Donor Group	
Patient's Group	First Choice	Second Choice
A+	0	None
B	0	None
AB	A or B+	0
A_2B with anti-A_1	A_2 or B+	0
A_1B with anti-'HI'(O)	A_1 or B+	None

* A patient with uninhabitable anti-'Hi'(O) should be transfused with blood of group A_1.

\+ Either group may be chosen, but only one of the two should be used for a given recipient. Group A is usually more readily available, than group B. If blood of yet another group is needed, use group O.

c. **Changing to Group-Specific Blood After Transfusion of a Different ABO Group.**

(1) The decision to change back to group-specific blood at any time is best based on the presence or absence of anti-A and/or anti-B in subsequent samples of the recipient's blood. Fresh blood specimens should be obtained on the day of each successive transfusion for evaluation. If crossmatches of a freshly drawn patient sample with group-specific blood indicate compatibility, this blood may be issued. Otherwise, transfusion with RBCs of a different ABO group should be continued. Group-specific transfusions should not be infused through the same infusion set as was used for transfusion of red cells of a different ABO group.

(2) When the emergency is over, the effect of transfused alloantibodies should be evaluated. Such antibodies may cause hemalysis of recipient cells. If Rh-negative blood is unavailable; transfuse Rh-positive blood, rather than withholding blood from a patient, whose need for blood is critical.

(3) Up to 70 percent of Rh-negative patients given Rh-positive blood may form anti-Rh_O(D). It is reasonable to attempt prevention of immunization with large doses of Rh_O(D) lmmune Globulin (Human) when only one unit of Rh-positive blood has been given accidentally. One dose (approximately 300 ug) of Rh_O(D) lmmune Globulin (Human) is required for 15 ml of RBCs; thus, 15 to 20 ml of Rh lmmune Globulin may be required to prevent immunization by one unit of blood. Rh-negative blood may be given to an Rh- positive recipient, if necessary.

1-46. MASSIVE TRANSFUSION

a. **Definition of Massive Transfusion.**

(1) Massive transfusion may be defined as rapid infusion of blood in amounts approaching or exceeding replacement of the recipient's total blood volume, within a 24-hour interval. This is encountered in surgical and medical emergencies in cardiac and vascular surgery, especially when extracorporeal circulation is used. The exchange transfusion of an infant is also a massive transfusion.

(2) An antibody present in the original recipient sample may be weakened or not detectable in a subsequent posttransfusion specimen because of the dilution effect of massive transfusion. It is especially important, in this case, to select blood that is negative for the corresponding antigen by typing the units with a reagent antiserum before crossmatching.

b. **The Planned Massive Transfusion.**

(1) Obtain a specimen of the patient's blood well in advance of the scheduled surgery for ABO, Rh typing, and antibody-detection test. Antibodies, detected, should be identified at this time. If hypothermia is to be used, special care must be given to identification of antibodies detected at room temperature.

(2) Obtain a sufficient amount of blood from the patient for the compatibility tests 72 hours or less before the scheduled transfusion. Frozen serum collected more than 72 hours prior is not adequate.

(3) Compatibility testing.

(a) Interdonor compatibility testing is not required. Donor units containing unexpected antibodies should not be used.

(b) If additional units are required within a 72-hour period, crossmatching may be done with the immediate pretransfusion sample. Any time, after that, a new sample must be obtained from the patient.

1-47. TECHNIQUES FOR CROSSMATCHING

a. **Background.**

(1) There are a variety of techniques available for crossmatching. Since some antibodies will react by certain methods but not by others, techniques should be chosen to include at least two incubation temperatures and media.

(2) Techniques considered optimal for hemolyzing and agglutinating antibodies include saline or serum suspensions of donor cells incubated with recipient serum at room temperature (18ºC to 25ºC) for 15 to 30 minutes. Although the detection of some agglutinating antibodies is improved by incubation at 4ºC, any advantage gained is outweighed by the finding of ubiquitous cold agglutinins, usually not significant in transfusion therapy. Hence, 4ºC incubation is not recommended for crossmatching.

(3) The optimal method for detecting coating antibodies is the use of an antiglobulin test following incubation at 37ºC for 15 to 60 minutes. Incubation may be carried out in a potentiating medium of high dielectric constant, such as albumin. The test is customarily read both before washing and after addition of antiglobulin serum.

b. **Technical Factors.** Technical factors must be considered in the performance of a crossmatch. These include:

(1) Donor RBCs, for crossmatching, must be obtained from a sealed segment of tubing integral with the container.

(2) The cells used for crossmatching may be saline-washed.

(3) A 2 to 5 percent cell suspension is usually recommended.

(4) Reaction tubes are generally 10 or 12 X 75 mm.

(5) The supernatant must be examined for hemolysis against a white background, before resuspending the centrifuged cells.

(6) An optical aid, such as magnifying lens, mirror, or microscope, is advised, but not necessary, for the reading of agglutination.

(7) Hemolysis, or agglutination, at any stage of the crossmatch indicates an incompatibility.

(8) The person performing the test should be familiar with incubation, centrifugation, antiglobulin technique, sources of error, reading of hemolysis, and agglutination.

(9) All test tubes should be labeled before use with unit and recipient identification.

NOTE: Two accepted procedures are given below; other techniques may also be acceptable.

c. **One-Tube Crossmatch Procedure.**

(1) STEP 1. Place two drops of recipient serum in a labeled tube.

(2) STEP 2. Add one drop of a 2 to 5 percent saline suspension of donor cells.

(3) STEP 3. Centrifuge; examine for hemolysis, agglutination; grade and record results.

NOTE: Cold agglutinins may cause difficulty with this procedure. The two-tube crossmatch may resolve the difficulty.

(4) STEP 4. Add 2 to 3 drops of 22 to 30 percent albumin according to manufacturer's directions; mix. Centrifuge, incubate 15 to 60 minutes at 37ºC, read and record the results.

(5) STEP 5. Centrifuge immediately upon removing from the incubator; examine for hemolysis, agglutination; grade and record results.

(6) STEP 6. Wash three or four times with saline. After last wash, decant completely. Add 1 to 2 drops of antiglobulin serum and mix.

(7) STEP 7. Centrifuge, examine for agglutination, grade and record the results. (Use of optical aid is optional at this step.)

(8) STEP 8. Add one drop of known sensitized cells to all negative tests. Centrifuge, examine for agglutination, and record result. If no agglutination is seen, the antiglobulin phase must be repeated. If no hemolysis or agglutination is seen in any phase, the blood is considered compatible.

d. **Two-Tube Crossmatch Procedure.**

(1) STEP 1. Place two drops of recipient serum in each of two labeled tubes.

(2) STEP 2. Add one drop of a 2 to 5 percent saline suspension of donor cells to each tube, and mix. Centrifuge, read, and record results.

(3) STEP 3.

(a) Incubate one tube 15 to 30 minutes at room temperature.

(b) Centrifuge; examine for hemolysis, agglutination; read and record results.

(c) Discard tube.

(4) STEP 4.

(a) The second tube may be left, as in Steps 1 and 2; add 2 to 3 drops of 22 to 30 percent albumin, according to manufacturer's directions; mix. Centrifuge, read and record results.

(b) Incubate the second tube 15 to 80 minutes, at 3º7C.

(c) Centrifuge immediately upon removing from the incubator. Examine for hemolysis, agglutination, read and record results.

(d) Wash 3 or 4 times with saline. After the last wash, decant completely. Add 1 to 2 drops antiglobulin serum and mix.

(e) Centrifuge; examine for agglutination with an optical aid; record results. (Optical aid is optional.)

(f) Add 1 drop of known sensitized cells to all negative test. Centrifuge; examine for agglutination record result. If no agglutination is seen, the antiglobulin test must be repeated.

e. **Immediate Spin Technique.** By AABB standards, minimal serological testing, (saline crossmatch), is an acceptable method to demonstrate ABO incompatibilities between the donor and the recipient if:

(1) Antibody detection is negative on the recipient, and/or,

(2) Recipient has no clinical significant antibodies.

1-48. INCOMPATIBLE CROSSMATCH

a. **Introduction.**

(1) When incompatibility is seen in an early phase of a cross-match, the testing should be completed to give information as to temperatures media where reactions occur, the variability of these reactions, and the percentage of incompatible donors. These clues will aid in choosing correct conditions for antibody identification.

(2) It is preferable to determine the cause of the incompatibility, rather than to continue blindly. If the need for transfusion is too urgent for this course of action, many random units may be crossmatched. If possible, attempts to identify the antibody should be started while the crossmatch is being completed.

b. **Work Outline for Incompatible Crossmatch.** Preliminary investigation: recheck ABO groups and Rh types of incompatible donors and recipients; recheck pilot sample numbers against donor units and run an autocontrol with the antibody identification panel.

c. **Autocontrol Negative.**

(1) Specific cold alloantibodies are suggested if reactions are detected at room temperature; if reactions are weak or negative at 37C, by antiglobulin technique; or if reactions are stronger at 18C to 15C, or lower. Under such conditions, do the following:

(a) Identify the antibody or antibodies.

(b) If anti-A_1 or anti-'HI'(O) is identified, select blood on the basis of A subgroups.

(c) If another specific antibody is identified, the RBCs of prospective donors should be tested for this antigen with known reagent antiserum, (if available), before transfusion. This is important because the antigenic strength varies among donors in many blood-group systems. The patient's antibody may not be strong enough to react with cells from units containing weak antigens. If antiserum is in short supply and the patient's serum plentiful, crossmatch several units first and use the antiserum to test only those units that appear compatible.

(2) Specific warm alloantibodies are suggested, if the reactions take place at 37C, and/or are detected by antiglobulin technique. Do the following:

(a) Identify the antibody or antibodies.

(b) Test the cells of prospective donors for the corresponding antigens with a known reagent antiserum.

(c) If the RBCs from only one donor unit react and the antibody screening test on the patient is negative, do a direct antiglobulin test on the donor. If the direct antiglobulin test is positive, quarantine the donor blood for further investigation. If it is negative, test the patient's serum for antibodies directed against low-incidence antigens.

(d) When RBCs from most donors react, there are two possible explanations. There may be an antibody against a high-incidence RBC antigen or multiple antibodies may be present. In either case, the patient's relatives, especially siblings, may be compatible. If not, it may be possible to find compatible donors through a Reference Laboratory, or Rare Donor File.

d. **Autocontrol Positive.**

(1) A cold antibody is suggested if reactions detected at room temperature are weak, or negative at 37ºC, and/or by antiglobulin technique, or if reactions are enhanced at 18ºC to 15ºC or lower. Reactions at 37ºC and by antiglobulin test may be a result of cold agglutinin if cells and serum were mixed at room temperature before incubating at 37ºC. The following procedure is suggested:

(a) If the patient has not been recently transfused, absorb cold autoagglutinins with patient's cells in an ice-slush bath.

(b) Autoabsorption should not be done if the patient has been recently transfused, because the transfused donor cells may absorb out a specific alloantibody; therefore, a prewarmed antiglobulin crossmatch should be done.

(c) Use absorbed serum for reverse grouping, crossmatching, and identification of alloantibodies that may have been masked by the cold antibody.

(d) Wash patient's cells with warm (3º7C) saline before grouping and typing.

(2) A serum protein abnormality in the patient may cause reactions that are detected at room temperature, enhanced at 3º7C, and negative by antiglobulin test. This pattern suggests a protein abnormality causing rouleaux formation, particularly when enhanced in albumin. The patient's diagnosis and results of protein tests can be informative here. More rarely, with a high-protein method, the albumin-agglutinating phenomenon may be present. The direct antiglobulin test is usually negative in this situation. If a protein abnormality is being considered, the following procedure is suggested:

(a) Use saline-reactive antiserum to Rh-type patient.

(b) Do a saline-antiglobulin test as the main index of compatibility, between donor and patient. See the appendix for compatibility testing in presence of strong rouleaux formation.

(c) Repeat albumin phase, using albumin without the capryilate stabilizer, when albumin-agglutinating phenomenon is suspected.

(d) Check donor's cells for polyagglutinability.

(3) A warm antibody is indicated if the reactions are detected at 37ºC, by antiglobulin technique, or by antiglobulin technique only. The direct antiglabulin test is usually positive with a warm autoantibody. Consider the following:

(a) Determine whether the autoantibody has demonstrable specificity or whether it may be masking an alloantibody.

1 Specific antibody may be seen with autoimmune hemolytic anemia or with alloimmunization and coating of transfused donor RBCs (the direct antiglobulin test may appear mixed-field). It is helpful to know the specificity (IgG or complement) of the protein coating the RBCs. If IgG is coating the RBCs, antibody can usually be eluted from the patient's cells; however, if only complement is coating the patient's cells, antibody will not be eluted. The results must be carefully interpreted.

2 When there is specificity, blood lacking the corresponding antigen should be selected for transfusion, if possible. Many specific warm autoantibodies are directed against Rh-Hr antigenic determinants.

3 When the specificity of antibody on the RBCs differs from that in the serum, it may be necessary to crossmatch with an eluate as well as serum.

4 Patients with autoimmune hemolytic disease may have a positive direct antiglobulin test, without demonstrable circulating antibody. This is because all antibodies have been absorbed onto the RBCs and, therefore, tests done with the patient's serum will appear compatible. To be more certain of compatibility, eluates prepared from the patient's coated cells may be used for crossmatching.

5 When antibody specificity cannot be determined, blood can be crossmatched by the titration technique. The patient's serum can then be absorbed with the cells of the weakest reacting donor and the absorbed serum tested for additional antibodies. The donor cell may also absorb out an underlying alloagglutinin. Responsibility for transfusion of blood that is incompatible "in vitro" should be shared by the blood bank physician, and the patient care physician.

(b) The patient cannot be phenotyped with antisera requiring the antiglobulin technique, because of the positive direct antiglobulin test. There may also be spontaneous agglutination with high-protein antiserum. Elution at 45°C before testing may help to remove enough antibody from the cells to allow typing.

e. **Some Technical Causes of Apparent Incompatibility.** False positive reactions may be caused by dirty glassware, bacterial contamination, chemical or other contaminants in reagents (including saline), fibrin clots, and overcentrifugation.

1-49. LABELING AND RELEASE OF CROSSMATCHED BLOOD

a. The prescribed compatibility record must be completed. A label or tag must be attached directly to each compatible unit of blood, and must remain attached, at least until the transfusion is completed. This label and any other accompanying forms must be completed.

b. The expiration date and appearance must be checked just before release of a unit of blood. Release forms should be compared with the blood label, request forms, and the release record must be filled out.

c. Final identification of the recipient and the blood container rests with the transfusionist, who must positively identify the patient and donor blood and compare the information with the compatibility report form.

1-50. RETENTION AND STORAGE OF BLOOD SAMPLES

The recipient's blood specimen and a donor sample must be sealed, or stoppered, and kept for at least 7 days following transfusion at 1ºC to 6ºC. If possible, the blood container should be returned to the blood bank, and stored for 24 hours or more. The Communicable Disease Center and the College of American Pathologists both require that the blood containers be returned and stored. This is not required by the AABB Standards.

1-51. RELEASE OF BLOOD IN EMERGENCY SITUATIONS

a. In an emergency, the patient's physician must weigh the risk of transfusing uncrossmatched or incompletely crossmatched blood against the hazard of waiting for completed crossmatch tests.

b. If the urgency of the situation warrants release of blood before the crossmatch is completed, the physician must indicate the urgent nature of the situation that requires the omission of the crossmatch. Such a release does not absolve the blood bank from its responsibility to issue properly grouped and labeled blood. In emergency situations:

(1) If necessary, issue uncrossmatched blood. Notify a blood bank physician that uncrossmatched blood has been released.

(a) If there is not time to determine the patient's blood group, issue group 0 Rh-negative blood that has most of the plasma removed or is free of hemolytic anti-A and anti-B. 0 Rh-positive blood may be issued only if 0 Rh-negative is not available.

(b) Group-specific blood should be given. If time permits, test the patient in the transfusion facility without relying on previous records. Evidence of the patient's blood group must not be taken from cards, dog tags, drivers' licenses, or other such records.

(2) Begin the routine compatibility testing procedure. If the clinician cannot wait the length of time required for a complete crossmatch, release the blood and continue the crossmatch after release.

(3) Complete the compatibility testing. If incompatibility is detected at any stage of the testing, immediately notify the patient's physician and the blood bank physician.

1-52. CROSSMATCH IN PRESENCE OF PROLONGED CLOTTING TIME

Blood samples from patients with coagulation abnormalities may not be fully clotted when serum and cells are separated. When such serum is incubated with donor RBCs, a fibrin web may trap masses of RBCs making it difficult to determine whether true agglutination has occurred. Coagulation of such blood specimens (except those deficient in fibrinogen) can usually be accelerated by the addition of thrombin to the patient's serum. One drop of thrombin (50 units/ml) per milliliter of serum, or the amount of dry thrombin that will adhere to the tip of an applicator stick, will convert the remaining fibrinogen instantly. Keep in mind that the addition of excess thrombin may cause nonspecific agglutination of all donor cells tested. If this occurs, additional serum must be obtained and glass beads may be added to help clot-formation. This should circumvent the nonspecific agglutination problem. If anticoagulated specimens are used, the donor cell suspension must be well washed to be certain that there is no carry-over of fibrinogen. It may be necessary to add one drop of protamine; however, protamine sulfate may cause rouleaux and, in excess, may also prolong clotting.

1-53. COMPATIBILITY TESTING AFTER INFUSION OF SYNTHETIC PLASMA EXPANDERS

Plasma expanders, such as some dextrans or other large molecular weight plasma substitutes, may cause rouleaux, and difficulty with compatibility tests. When fresh serum that was collected before the infusion is not available, the saline replacement technique should be performed. The saline-antiglobulin test will usually be compatible; the high protein test may appear incompatible.

Continue with Exercises

EXERCISES, LESSON 1

INSTRUCTIONS: Answer the following exercises by marking the lettered response that best answers the exercise, by completing the incomplete statement, or by writing the answer in the space provided at the end of the exercise.

After you have completed all the exercises, turn to "Solutions to Exercises" at the end of the lesson and check your answers. For each exercise answered incorrectly, reread the material referenced with the solution.

1. What are the smaller molecules called that have a molecular weight of less than 4,000, and can be immunogenic if they are coupled with larger proteins?

   a. Haptens.

   b. Alleles.

   c. Antigens.

   d. lmmunoglobulins.

2. When an antigen enters the body, the response may be humoral or:

   a. Delayed.

   b. Cellular.

   c. lmmediate.

   d. Anaphylactic.

3. Cells that excrete antibodies are:

   a. Monocytes.

   b. Neutrophils.

   c. 8-lymphocytes.

   d. T-lymphocytes.

4. The lag period after first exposure to an antigen before circulating antibody can be detected is:

    a. 5 hours.

    b. 5 days.

    c. 5 weeks.

    d. 5 months.

5. The anamnestic response is most closely related to the concept of:

    a. Homeostasis.

    b. Innate immunity.

    c. Cellular immunity.

    d. Immunologic memory.

6. What is the preferred term for antibodies to blood group antigens other than A or B?

    a. Abnormal

    b. Atypical.

    c. Irregular.

    d. Unexpected.

7. The antibody's rate of bonding to the red cells should increase as the ionic strength of the reaction medium is:

    a. Reduced.

    b. Increased.

8. What is an antibody (often lgG) called that reacts with a saline suspension of red blood cells, without producing a visible reaction?

    a. A blocking antibody.

    b. An incomplete antibody.

    c. A despeciated antibody.

    d. A neutralizing antibody.

9. Which antlglobulin test is used in diagnosis of hemolytic disease of the newborn and autoimmune hemolytic anemia?

    a. Direct.

    b. Indirect.

10. Which antiglobulin test is used in crossmatching, detection of unexpected antibodies, and detection of antigens not identifiable by other means?

    a. Direct.

    b. Indirect.

11. What is the reason for a false negative result if an inadequate washing of cells for antiglobulin test is done?

    a. Complement blocks the action of antiglobulin.

    b. Contaminants block the reaction sites of red cell antibodies.

    c. Trace amounts of residual globulin neutralize antiglobulin serum.

    d. The electronegativity of the red cells repels the antiglobulin molecules.

12. Pooling reagent red cells is **NOT** recommended except when screening the sera of:

    a. Blood donors.

    b. Jaundiced newborn.

    c. Recipients after transfusion reactions.

    d. Recipients after incompatibility in crossmatching.

13. Which procedure is included in the antibody screening?

    a. Reaction in saline.

    b. Indirect antiglobulin test.

    c. Reaction at 37ºC with albumin or other potentiating medium.

    d. All of the above.

14. What is the incubation time involved in each of the first two numbered procedures of the antibody screening test?

    a. 2-5 minutes.

    b. 15-30 minutes.

    c. 1 hour.

    d. 2 hours.

15. What factor is useful when preparing to identify antibodies detected in the antibody-screening test?

    a. History of drug therapy.

    b. Results of previous testing.

    c. Results of direct antiglobulin test.

    d. History of transfusion or pregnancy.

    e. All the above.

16. A reagent RBC panel for antibody identification should include as many as possible of the common _____ and provide a distinct pattern of reaction for single _____ to most of the _____ represented on the panel.

    a. Antigens, antigens, antibodies.

    b. Antigens, antibodies, antigens.

    c. Antibodies, antigens, antibodies.

    d. Antibodies, antibodies, antigens.

17. When a patient's red cells lack the corresponding antigen, what antibody is indicated by the results with the red cell panel? Use Exercise figure 1-1 shown below.

    a. Anti-C.

    b. Anti-E.

    c. Anti-Le^a.

    d. Anti-Fy^a

	ANTIGENIC DETERMINANTS																		RESULTS			
Cell	D	C	E	c	e	C^w	K	k	Fy^a	Fy^b	Jk^a	Jk^b	Le^a	Le^b	S	s	M	N	P_1	Lu^a	Lu^b	Xg^a	22°C sal	37°C alb	A T	G
1	+	+	0	0	+	+	0	+	+	+	+	0	+	0	+	0	+	+	0	+	0	0	0	0	0	0
2	+	+	0	0	+	0	+	+	+	+	+	0	+	0	+	+	0	+	0	+	+	0	0	0	0	0
3	+	0	+	0	+	0	+	0	+	+	0	+	+	0	+	+	+	+	0	+	+	0	0	2+	2+	2+
4	0	+	0	+	+	0	+	+	+	+	+	+	+	0	+	0	+	+	0	+	0	0	0	0	0	0
5	0	0	+	+	+	0	+	+	0	+	0	+	0	+	0	+	+	+	0	+	0	0	0	2+	2+	2+
6	0	0	0	+	+	0	+	+	0	+	+	0	0	+	0	+	0	+	0	+	+	0	0	0	0	0
7	0	0	0	+	+	0	+	+	0	+	+	0	0	+	0	+	0	+	0	+	+	0	0	0	0	0
8	0	0	0	+	+	0	+	+	0	+	+	0	0	+	0	+	+	+	0	+	+	0	0	0	0	0
Autologous red cells																							0	0	0	0

Exercise Figure 1-1. Antigenic determinant results for Exercise 17.

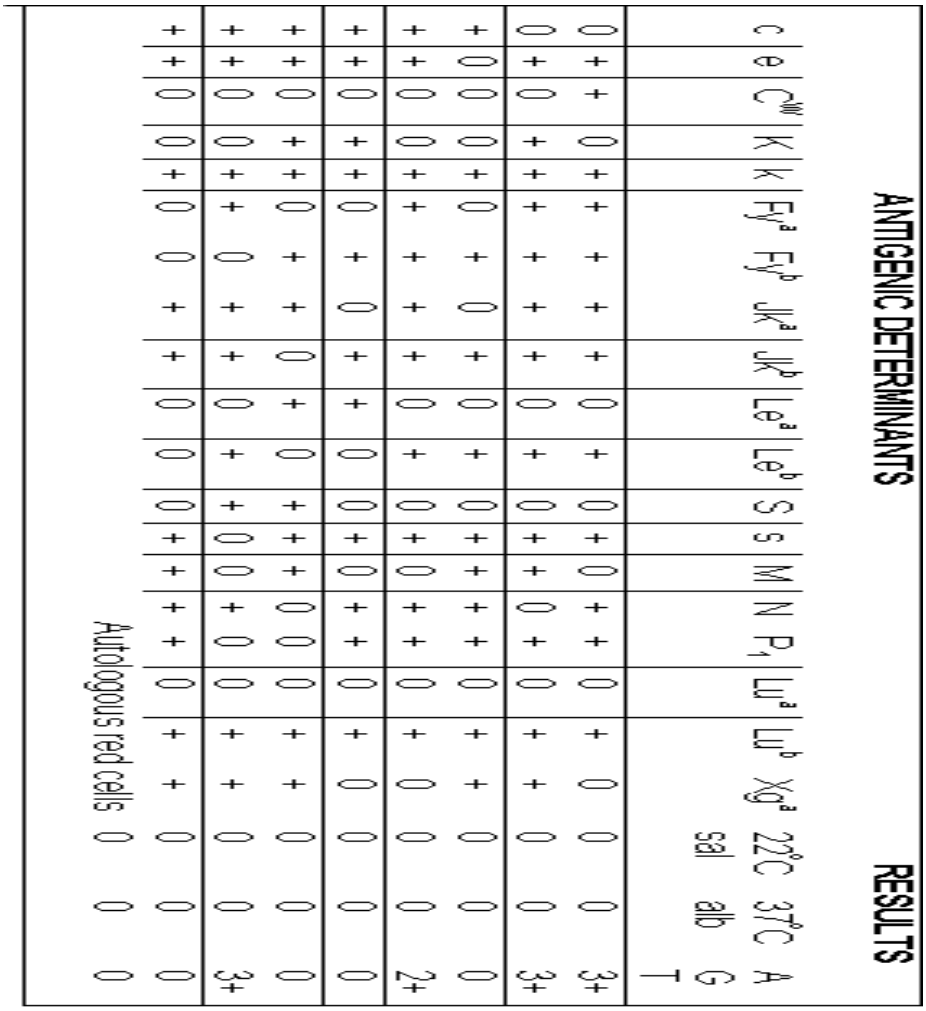
18. When the patient's red cells lack the corresponding antigen, what antibody is indicated by the results with the red cell panel? Use exercise figure 1-2 shown below.

    a. Anti-E.

    b. Anti-D.

    c. Anti-Le^{a}.

    d. Anti-Fy^{a}



Exercise Figure 1-2. Antigenic determinant results for Exercise 18.

19. What do you suspect if an antibody reacts with several red cell suspenslons on a panel without giving a specific pattern and reacts with red cells of 65 percent of random donors?

    a. Anti-D.

    b. Anti-Lu^{a}.

    c. Anti-Do^{a}.

    d. Anti-Vell.

20. Rouleaux are:

    a. Opaque.

    b. Inflexible.

    c. Irregular in outline.

    d. Dispersable with saline.

    e. All the above.

21. What do you suspect, when the antibody-screening test demonstrates activity at room temperature (at 37ºC) and in the antiglobulin test portion?

    a. Cold antibodies only.

    b. Warm antibodies only.

    c. Both cold and warm antibodies.

    d. Neither cold nor warm antibodies.

22. AntIbodies that may demonstrate a dosage effect, dependent upon the zygosity of an antigenic determinant, include:

    a. Anti-M,

    b. Anti-N.

    c. Anti-Jk^a.

    d. AntI-Jk^b.

    e. All the above.

23. In the procedure for autoabsorption of cold-reacting antibodies, the blood with anticoagulant is kept at _____ to supply the _____ for testing.

    a. 37ºC, cells.

    b. 37ºC, serum.

    c. About 4ºC, cells.

    d. About 4ºC, serum.

24. When the serum still contains cold-reacting autoantibodies after the initial cold incubation in the autoabsorption procedures, it is incubated with autologous cells for _____ at _____.

    a. 2 hours, 37ºC.

    b. 2 hours, about 4ºC.

    c. 30-60 minutes, 37ºC.

    d. 30-60 minutes, about 4ºC.

25. What is standardized in the titration of antibodies?

    a. Antibody.

    b. Reagent cells.

    c. Reaction conditions.

    d. All the above.

26. Titration of antibodies is used in blood banking to:

    a. Identify anti-I activity.

    b. Identify the "least incompatible" donor units.

    c. Demonstrate changes in antibody levels during pregnancy,

    d. All the above.

27. The text suggests that a large-volume dilution may be _____ than the 0.1 ml technique for titrations.

    a. Easier.

    b. Less accurate.

    c. More difficult.

    d. More time-consuming.

28. Absorption is removal of an antibody from:

    a. Serum.

    b. Red blood cells.

29. Elution is removal of antibody from:

    a. Serum.

    b. Red blood cells.

30. Before crossmatching, what should be known about the recipient?

    a. Rh type.

    b. AB0 group.

    c. Results of previous testing.

    d. Results of antibody screening test.

    e. All the above.

31. Upon whom is the "major crossmatch" performed?

    a. The donor's cells and the donor's serum.

    b. The recipient's serum and the donor's cells.

    c. The recipient's serum and the donor's serum.

    d. The recipient's cells and the donor's serum.

32. The person performing a crossmatch procedure realized the donor was group O and the recipient was group A with no evidence of unexpected antibodies. At which phase of the procedure would you expect to see a react ion?

    a. When the albumin is at 37ºC.

    b. Immediately during the spin of saline.

    c. Immediately during the spin of the albumin.

    d. None of the above.

33. A major crossmatch will detect:

    a. Most Rh typing errors.

    b. All ABO grouping errors.

    c. All unexpected antibodies.

    d. Antibodies in the recipient's serum that react with antigens on the donor's red cells.

    e. All the above.

34. Great care must be taken during collection of the recipient's blood to ensure positive identification of the:

    a. Donor's ABO group and Rh type.

    b. Donor and donor unit of blood.

    c. Patient's ABO group and Rh type.

    d. Patient and patient blood sample.

35. What must be done during pretransfusion testing if a discrepancy is noted between the blood request form and the label on the recipient's blood sample?

    a. Cancel the transfusion.

    b. Draw a new blood sample.

    c. Relabel the blood sample.

    d. Rewrite the blood request form.

36. For pretranfusion testing, the recipient's serum should be less than _____ days old to help ensure the presence of complement.

    a. 3.

    b. 4.

    c. 6.

    d. 8.

37. Units of blood held for several days must be recrossmatched with a new serum specimen if the last transfusion given was over _____ hours.

    a. 12.

    b. 24.

    c. 48.

    d. 72.

38. The donor and recipient should have the same ABO group and Rh type **EXCEPT** when there is:

    a. Unavailability of such blood.

    b. ABO or Rh hemolytic disease.

    c. All the above.

39. After transfusion of nongroup-specific blood, changing to group-specific blood is permitted only if crossmatches with a freshly drawn patient sample indicate:

    a. Compatibility.

    b. Incompatibility.

40. In the two-tube crossmatch procedure, the first incubation temperature is _____ and the second incubation temperature is _____.

    a. 37ºC; 4ºC.

    b. 40ºC; 37ºC.

    c. About 22ºC; 37ºC.

    d. 37ºC; about 52ºC.

41. The crossrnatch includes:

    a. A 37ºC phase.

    b. An antigiobulin serum phase.

    c. A room temperature phase (saline).

    d. All of the above.

42. After addition of sensitized cells to a negative crossmatch, the antiglobulin test must be repeated if there is:

    a. Agglutination.

    b. No agglutination.

43. If there is no time to determine the patient's blood group, group 0 Rh-negative blood may be given if:

    a. Most of the plasma is removed.

    b. An emergency release form is signed.

    c. All the above.

44. Once uncrossmatched blood has been released for transfusion, you should _____ the crossmatch.

    a. Complete.

    b. Discontinue.

45. To accelerate clot formation in the patient's blood specimen, add:

    a. Calcium.

    b. Heparin.

    c. Thrombin.

    d. Fibrinogen.

Check Your Answers on Next Page

SOLUTIONS TO EXERCISES: LESSON 1

1. a (para 1-3c)
2. b (para 1-4a)
3. c (para 1-4d)
4. b (para 1-5a)
5 d (para 1-5b)
6. d (para 1-6c)
7. a (para 1-9b(4)(b))
8. b (para 1-9c(1))
9. a (para 1-16b(1) and (2))
10. b (para 1-17b)
11. c (para 1-24a(1))
12. a (para 1-28b(2))
13. d (para 1-26)
14. b (para 1-26a)
15. e (para 3-32(b))
16. b (para 1-28c)
17. b (para 1-33)
18. d (para 1-33)
19. c (para 1-36e)
20. d (para 1-37a)
21. c (para 1-37d)
22. e (para 1-37e(1))

23. a (para 1-38b(2) Step 1 and 2)

24. d (para 1-38b(2) Step 6 and 7)

25. d (para 1-39a(2))

26. d (para 1-39a(2))

27. a (para 1-39d)

28. a (para 1-40a(1))

29. b (para 1-41a)

30. e (paras 1-42, 1-43, 1-45)

31. b (para 1-42c)

32. d (para 1-42d)

33. d (para 1-42e)

34. d (para 1-43b)

35. b (para 1-44)

36. a (para 1-44a(1))

37. d (para 1-44a(2))

38. c (paras 1-45a(1)(a), (b))

39. a (para 1-45c(1)

40. c (para 1-47c, Step 3 and 4)

41. d (para 1-47)

42. b (para 1-47d Step 4f)

43. c (para 1-51b(1)(a))

44. a (para 1-51b(2))

45. c (para 1-52)

End of Lesson 1

LESSON ASSIGNMENT

LESSON 2	Immune Hemolytic Anemia and Hemolytic Diseases of the Newborn
TEXT ASSIGNMENT	Paragraph 2-1 through 2-22.
LESSON OBJECTIVES	After completing this lesson, you should be able to:

2-1. Identify the elements in the investigation of a positive direct antiglobulin test and diagnostic procedures for immune hemolytic anemia.

2-2. Identify the factors in hemolytic disease of the newborn (HDN), including causes, mechanisms, prenatal studies, neonatal studies, clinical features, and criteria for exchange transfusions and RhIG prophylaxis.

SUGGESTION After completing the assignment, complete the exercises at the end of this lesson. These exercises will help you to achieve the lesson objectives.

LESSON 2

DISEASES OF THE NEWBORN

Section I. INVESTIGATION OF A POSITIVE DIRECT ANTIGLOBULIN TEST AND IMMUNE HEMOLYTIC ANEMIA

2-1. GENERAL

a. Some of the causes of positive direct antiglobulin tests have been discussed in Lesson 1, Section II. The results of a direct antiglobulin test should reflect what is happening "in vivo," not what has occurred "in vitro," through use of inappropriate techniques (for example, cold autoantibodies or complement sensitizing the RBCs at room temperature or 4ºC).

b. It is important to note that “in vivo” RBC sensitization does not necessarily mean an individual has autoimmune hemolytic anemia or even that an autoantibody has caused the positive reaction. "In vivo” RBC sensitization can be associated with any of the following situations.

(1) Alloantibodies present in the recipient’s plasma may sensitize transfused RBCs. Alloantibodies present in the donor’s plasma can also sensitize recipient’s RBCs. Alloantibodies crossing the placenta may sensitize fetal RBCs.

(2) Autoantibodies can react with intrinsic RBC antigens, leading to sensitization of the RBCs with immunoglobulins and/or complement components. Hemolytic anemia may or may not be present.

(3) Antibodies against drugs may be formed, for example, penicillin antibodies. These antibodies may sensitize drug-coated RBCs.

(4) Red blood cells may take up proteins through nonimmunologic processes. This has been shown to occur following exposure to drugs of the cephalosporin group where the RBCs membrane becomes chemically altered so that many proteins are nonspecifically absorbed to the membrane.

(5) Immune complex formation. See Lesson 1, Section II and later in this lesson.

c. In the serologic investigation of “in vivo” RBC sensitization, it is important to exclude first the “in vitro” causes of false-positive reactions mentioned earlier. The most common pitfall is to interpret the positive results obtained on cells from a refrigerated clot as indicating in ” vivo” sensitization. Before such a positive result is accepted, the test must be repeated on a fresh blood sample, preferably taken into EDTA, to prevent “in vitro" complement uptake. See Lesson 1, Section II on causes of false-positive results.

d. The following section details the investigations used to help diagnose an immune etiology for a hemolytic anemia, but the techniques employed can be used for the investigation of any positive direct antiglobulin test. The specific investigations for "in vivo" sensitization associated with incompatible blood transfusions and hemolytic disease of the newborn are dealt with in Lesson 2, Section II and Lesson 3, SectIon II.

2-2. IMMUNE HEMOLYTIC ANEMIA

a. Immune hemolytic anemia is a state in which a patient has a shortened RBC survival associated with hemolysis resulting from an immune reaction. There are many causes for anemia, one of which is hemolysis. Furthermore, there are many causes for hemolytic anemia, and one involves an immune mechanism. The serologic investigations carried out in the blood bank do not determine whether a patient has hemolytic anemia; this is decided by the clinical findings and laboratory data such as hemoglobin, hematocrit, reticulocyte count, RBC morphology, bilirubin, haptoglobins, LDH, and possibly isotope studies. The serologic results do help decide if the hemolysis has an immune basis, and if it does, what type of immune hemolytic anemia is present? This is important since the treatment for each type is very different.

b. Immune hemolytic anemia may be classified in various ways. Common classifications based on serologic findings are as follows:

(1) Autoimmune hemolvtic anemia (AIHA).

(a) AIHA associated with warm antibodies.

1 Primary (idiopathic).

2 Secondary (lymphoma, SLE, Infections, carcinoma, and so forth).

(b) AIHA associated with cold antibodies.

1 Cold agglutinin syndrome.

a Primary (idiopathic).

b Secondary (lymphoma, mycoplasma pneumonia, infectious mononucleosis).

2 Paroxysmal cold hemoglobinuria.

a Primary (idiopathic).

b Secondary (syphilis, viral infections).

(2) Drug-induced hemolvtic anemia.

(3) Alloimmune hemolytic anemia.

(a) Hemolytic disease of the newborn.

(b) Hemolytic transfusion reactions.

NOTE: This Section will not deal with the alloimmune hemolytic anemias. See Lesson 2, Section II and Lesson 3, Section II.

c. In one series of 200 patients with immune hemolytic anemia, 66 percent had AIHA associated with warm antibodies, 16 percent had cold agglutinin syndrome, 2 percent had paroxysmal nocturnal hemoglobinuria, and in 16 percent the anemia was drug-induced. Other reported series are similar.

2-3. GENERAL SEROLOGIC INVESTIGATIONS

Before any serologic tests are performed, it is important to collect blood samples for the investigations in an appropriate manner.

a. **Collection of Blood Sample.** Collect clotted blood to provide serum and blood in EDTA, to provide RBCS for the direct antiglobulin test, grouping, and preparation of eluates. If it is not known whether the patient has AIHA associated with warm or cold antibodies, it is preferable to collect blood and separate it at 37ºC. If powerful cold autoantibodies are present, several problems can arise if the blood is not kept at 37ºC.

(1) Autoagglutination of RBCS will occur leading to difficulties in grouping.

(2) There will be a loss of antibody from the serum by auto-absorption, leading to false low cold agglutinin titers, and so forth.

(3) "In vitro" complement autosensitization may occur if EDTA blood is not used, leading to false increase in the strength of the direct antiglobulin test. EDTA will prevent any "in vitro" complement sensitization, even if the blood is cooled; thus, any complement detected on the RBCS represents “in vivo" sensitization.

(4) Possible hemolysis of the RBCS may occur "in vitro" utilizing antibody and complement, again leading to false low laboratory values and possible misinterpretation of “in vitro” hemoglobinemia, as being an "in vivo” occurrence.

b. **Serology.** To diagnose and classify the patient correctly, several questions have to be answered:

- **QUESTION:** Are the patient's cells sensitized with protein?

- **EXPLANATION:** The patient's washed RBCSs are first tested with a broad-spectrum antiglobulin reagent. Most commercial ant globulin serums will detect lgG sensitization adequately, but approximately 30 percent of all AIHA cases have only complement (C3d and C4d) on their RBCs and very rare cases have been described with only lgA or lgM on their cells. Some commercially prepared antiglobulin serums will not reliably detect these proteins; others, will only detect the sensitization if the antiglobulin serum and the sensitized red cells are incubated at room temperature for 5 to 10 minutes before centrifugation and reading. A single negative direct antiglobulin test should not be considered definitive evidence against a diagnosis of AIHA in a patient with suggestive signs and symptoms. Other rare cases exist in which the number of lgG molecules on the cells is below the threshold of the antiglobulin test and AIHA exists in the presence of a negative direct anti-globulin test.

- **QUESTION:** What proteins are present on the RBCs?

- **EXPLANATION:** The RBCs are often sensitized with lgG and complement, or IgG alone, and sometimes, by complement alone. The presence of these proteins is best detected by performing antiglobulln tests using monospecific antiglobulin serums, such as anti-lgG and anticomplement. lgA and lgM are sometimes detected but they are usually present together with IgG and/or complement.

NOTE**:** A word of caution should be added concerning monospecific antlglobulin reagents. At present only anti-IgG and anti-C3 are available as a licensed reagent, for use with RBCs. Other antiserums (for example., anti-IgA, -lgM, -C4) are readily available as precipitating reagents for use, in such techniques, as immunoelectrophoresis, but are not so readily available for use with RBCs. The precipitating reagents can be used as long as they are carefully standardized and controlled, (for example, they often contain antispecies agglutinins, which, have to be removed either by dilution, orabsorption with nonsensltized human RBCs). The quality assurance must be precise, as agglutination is a far more sensitive technique than precipitation, and monospecificity by precipitation techniques, does not ensure monospecificity by the antiglobulin test. It is preferable to obtain reagents specifically standardized for use with RBCs.

- **QUESTION:** Does the serum contain antibodies?
  Are they agglutinating or nonagglutinating (incomplete)?
  Do they show hemolytic activity?
  At what temperature do they react optimally?
  What is their thermal range?
  What is their specificity?
  Are they autoantibodies or alloantibodies?

- **EXPLANATION:** These questions can be answered by the regular serologic techniques used in the blood bank for detection and identification of antibodies. A few minor modifications are useful.

  - As patients with immune hemolytic anemia often have low serum complement levels, it is advisable to set up a duplicate set of tests, to which an equal volume of fresh compatible inert serum has been added, as a source of complement. Some workers prefer the mixture of patient's serum and complement to be in the pH range of 6.5 to 6.8, which is achieved by adding a one-tenth volume of 0.2 M HCl to the serum, as this seems to be optimal for the detection of warm and cold hemolysins.

  - Both warm and cold reacting autoantibodies give enhanced reactions with enzyme-treated RBCs; thus, it is useful to include such cells in the serum-screening procedure. Hemolysis of enzyme-treated cells is much more commonly observed, than hemolysis of untreated RBCs.

  - It is extremely important that tests, set up at 37ºC, are strictly at 37ºC. In order to do this, the patient's serum, reagent red blood cells, and albumin are warmed to 37ºC separately before mixing. The tests are then centrifuged at 37ºC (see later), and the cells are washed at 37ºC with 37ºC saline for the antigiobulin test. If this is not done carefully, (with careful control of the temperature at each stage), positive results may be misinterpreted, leading to possible confusion in the classification of the immune hemolytic anemia, and clinical significance of the autoantibody. This is particularly important when antibodies reactive at room temperature are present.

c. **Specificity of the Antibodies Eluted from the Patient's Red Blood Cells and in the Serum.**

(1) Red blood cell eluate. It is essential to prepare an eluate from the RBCs in order to define the autoantibody. If the positive direct antiglobulin test is a result of sensitization with complement components only, no antibody is usually detectable in the eluate, from the RBCs. If lgG is present on the cells, it can be eluted by simple methods, (for example, heat or ether). The eluate should contain autoantibody only, (this can often be confirmed by showing the presence of the matching antigen on the patient's RBCs). If the direct antiglobulin test Is positive, as a result of lgG sensitization, and the eluate shows no activity against normal cells, an association with drugs should be strongly suspected. A good example of this is penicillin-induced hemolytic anemia, where, the eluate from a strongly positive direct antiglobulin test will not react with any untreated normal cells; however, it will react strongly with the same cells treated with penicillin.

(2) Serum.

(a) The serum may contain:

1 No antibodies; they may all have been autoabsorbed onto the patient's RBCs "in vivo".

2 Autoantibodies only.

3 Autoantibodies plus alloantibodies.

4 Alloantibodies only.

(b) Comparing the specificity of antibody present in an eluate from the RBCs with antibody in the serum helps indicate the presence of alloantibody in the serum. The patient's RBC phenotype may help to confirm this.

(c) The specificity of the autoantibodies associated with warm antibody AIHA is very complex. The main specificity is directed against the Rh complex, but may only be obvious if rare cells, such as -D-, Rh_{null}, LW-negative, U-negative, and Wr^b-negative are available. If these cells are available, one can demonstrate "Rh" specificity in the broadest sense, in approximately 70 percent of these cases. Clear-cut specificity, such as anti-Rh_O(D), rh'(C), rh"(E), hr'(c), and hr"(e) is present only rarely, anti-hr"(e), being the most common. Usually, the undiluted eluate or serum will react with all RBCs of common Rh phenotype, but variations in strength of reaction may be noted.

2-4. SPECIFICITY OF AUTOANTIBODY

a. **Warm Autoimmune Hemolytic Amenia.** The specificity of autoantibodies associated with warm autoimmune hemolytic amenia (WAIHA) is very complex. Specificity is often directed against the Rh antigen complex, but this can only be determined if RBCs of very rare phenotypes are available, such as -D-or Rh null. Apart from Rh specificity, there have been reports of warm autoantibodies with anti-A, En^a, -Ge, $-I^t$, $-JK^a$, -K1, -K4, -K5,-K13. -Lan, -LW, -Nn, -Sci, -U, $-Wr^b$, and Xg^a specificities. It is seldom necessary to perform additional testing to determine the autoantibody specificity and then to select antigen-negative blood for transfusion. In some patients, simple specificity will be readily apparent (for example, autoanti-e). When there is evidence of immune hemolysis and the simple autoantibody specificity is clear-cut-not-relative, there may be some benefit in providing antigen negative blood. However, it is important not to expose patients to Rh antigens that their own cells lack, especially D. In instances where apparent specificity is directed to a high-incidence antigen or when the autoantibody fails to react with red cells of an uncommon Rh phenotype (for example, -D- Rh null), the compatible donor blood is unlikely to be available for transfusion. Thus, there is no point to determine specificity in the first place. If such blood is available, it should be reserved for alloimmunization (antibodies are developed in response to antigens from genetically dissimilar individuals of the same species) patients of that uncommon phenotype.

b. **Specificity of Cold Aggutinins.** If the patient has cold autoaggautinins, the specificity is usually within the Ii system. In addition to testing the serum against a cell panel, the serum is titrated against adult I cells, cord i cells, and, if possible, adult i cells at various temperatures (for example, 4ºC, 30ºC, or 37ºC). In this way, a cold agglutinin titer specificity and thermal range are determined using one set of master dilutions.

2-5. COLD AGGLUTININ TITER. THERMAL AMPLITUDE, AND CONFIRMATION OF ANTI-I(i) SPECIFICITY

a. Procedural notes are to be followed.

(1) A master series of doubling dilutions of serum is made in isotonic saline from 1:1 to 1:8,000 (approximately 0.2-ml aliquots).

NOTE: It is extremely important to use separate pipettes for each tube when preparing the dilutions to prevent “carry over." If a single pipette is used throughout, falsely high results occur (for example, a titer of 1:100,000 may only be 1:4,000 when separate pipettes are used).

(2) Starting from the highest dilution, two drops are transferred to three 10- X 75-mm tubes.

(3) The tubes are placed in a 37ºC water bath and allowed to reach 37ºC (for example, 10 minutes).

(4) One drop of 37ºC prewarmed 2 to 5 percent RBCs is added to each tube. To row 1, add group O I adult cells; to row 2, group O i cord cells, and to the third row, the patient's own cells. If adult i cells are available, a fourth row of dilutions is necessary.

(5) The tubes are incubated for 30 to 60 minutes and centrifuged at 37ºC. The tubes are kept at 37ºC while reading one tube at a time rapidly, macroscopically. Because most laboratories find it difficult to centrifuge accurately at 37ºC, a settling technique may be necessary. The tubes are incubated for at least two hours and one tube read at a time as before, but without centrifugation.

(6) After reading and resuspension of the cells, the tubes are then moved to the next temperature (for example, 30ºC), and the procedure repeated. At 30ºC, a settling technique (see paragraph above) will almost certainly have to be used.

(7) The tubes are finally incubated at 4ºC. Following 30 minutes to 1 hour at 4ºC, they are centrifuged at 4ºC. If a refrigerated centrifuge is not available, the cells can be left to settle for at least 2 hours (even overnight) and the tubes read macroscopically without centrifugation.

NOTE: It is very important that the tests be maintained at the particular temperature at all stages (for example, when removing tubes from the 4ºC refrigerator or refrigerated centrifuge), place the rack of tubes in a container of melting ice while reading individual tubes as rapidly as possible. If this is not done, the cold agglutination may disperse, leading to low false values.

b. Cold autoagglutinins may be present up to a titer of 64, at 4ºC, in healthy individuals.

c. Cold autoabsorptions will have to be performed to detect any cold alloantibodies (Lesson 3, Section III).

2-6. ABO AND Rh GROUPING

a. **ABO.**

(1) There should be no problems in ABO and Rh typing patients with AIHA if certain rules are followed; however, if they are not followed, serious errors can occur.

(2) The blood samples should have been collected and separated initially at 37ºC. The cells should be washed at 37ºC with warm saline. The cells are tested in the usual fashion with anti-A, anti-B, and anti-A, B, but a negative control of 5 to 10 percent albumin is also set up. This control should be compared with the tests, and if positive, indicates either non-dispersed autoagglutination or spontaneous agglutination of heavily sensitized cells in albumin.

(3) The patient's serum is tested against A_1, B, O, and his own cells, as usual. If the patient is known to have, or appears to have, the cold-agglutinin syndrome, the serum-typing tests should be repeated strictly at 37ºC. At this temperature, ABO agglutinins will usually react well, but the cold autoantibody will not react. These results should be confirmed by the normal methods after the cold autoantibody has been autoabsorbed from the patient's serum (Lesson 1, Section III).

b. **Rh.** It is useful to Rh-phenotype patients with AIHA. If the cells have been separated and washed at 37ºC, the main problem is that the cells are possibly strongly sensitized (perhaps with Rh autoantibodies) and will agglutinate spontaneously with the addition of albumin alone; therefore, when using Rh-typing serums, it is wise to use antiserums for saline tube test. Unfortunately, many commercial companies add a small amount of albumin (normally 5 to10 percent) and/or other potentiating media to such serums and, therefore, patient's cells should always be added to albumin (for example, 10 percent) as a negative control. An ideal negative control is the actual diluent that the manufacturer has used to dilute the antiserums, but this is not readily available for saline tube test reagents. If the albumin antiserums have to be used (for example, slide and rapid tube reagents), it is important that the diluent supplied by the same manufacturer of the antiserum be used as a negative control. If this is not available, then 20 to 30 percent albumin should be used as a control.

2-7. AUTOIMMUNE HEMOLYTIC ANEMIA ASSOCIATED WITH WARM ANTIBODIES

a. **Background.** This is the most common type of autoimmune hemolytic anemia (AIHAI). It comprises approximately 66 percent of all cases. There are typical serologic findings in warm antibody AIHA as noted below.

b. **Direct Antiglobulln Test.** If anti-lgG and anticomplement monospecific antiglobulin serums are used, three patterns are found, the most common being RBCs sensitized with both lgG and complement (about 50 percent of the patients); then; approximately, 30 percent are sensitized with lgG alone and 20 percent with complement alone. Sometimes lgA, and/or lgM can also be demonstrated on the RBCs and rare cases have been described where only lgA or lgM is present.

c. **Eluate From Patient's Red Blood Cells.** The autoantibody is usually lgG and can be eluted easily by simple methods, such as heating to 56ºC or treatment with ether. It is essential to prepare an eluate in the investigation of AIHA in order to define the characteristics of the autoantibody. It should be remembered that if the positive direct antiglobulin test is a result of complement sensitization only, the eluate will probably have no activity.

d. **Serum.** As the autoantibody reacts optimally at 37ºC and thus is being absorbed “in vivo," the patient’s serum may contain very little free antibody. Generally speaking, free autoantibody may only appear in the serum when all antigen sites on the patient’s RBCs are filled and no more antibody can be absorbed (for example, when the direct antiglobulin test is strongly positive). Thus, only approximately 35 percent of serums from warm antibody AIHA will react by the indirect antiglobulin test. If more sensitive techniques, such as, the use of enzyme-treated RBCs, are employed, over 90 percent of the serums can be shown to contain autoantibody. The antibody may be IgG and/or IgM and react optimally at 37ºC. Agglutinins and hemolysins against untreated RBCs at 37ºC are extremely rare, but warm hemolysins, active only against enzyme-treated RBCs, occur in about 20 percent of the serums. Although cold agglutinins titers are within the normal range (for example, up to 64) at 4ºC, it is not unusual to find them reacting up to room temperature in serums from patients with warm antibody AIHA. It is important to remember that the serum may contain alloantibodies in addition to autoantibodies or quite often only alloantibodies, the autoantibody having been absorbed by the patient’s RBCs “in vivo.”

e. **Specificity.** The specificity of the autoantibodies associated with warm antibody AIHA is very complex. As discussed previously, the main specificity is directed against the Rh complex, but may only be obvious if very rare cells such as, -D-, or Rh_{null}, are available. Apart from Rh specificity, there have been reports of anti-U, anti-LW, anti-I^T, anti-Kell, and anti-Wr^b, being associated with warm antibody AIHA.

f. **Compatibility Testing.**

(1) Blood transfusions should be avoided in AIHA. The transfusion of incompatible blood, especially in the presence of alloantibody that may be masked by “nonspecific” autoantibody, involves substantial risk. In order to assume these risks, there must be clinical needs sufficiently great to overcome the potential danger. It is equally wrong to avoid all transfusions unequivocally because of incompatible blood while a patient dies with progressive anemia. Clinical judgment must resolve these problems.

(2) There are two main problems to be considered before carrying out compatibility tests on AIHA patients. The first, and most important, is to determine if alloantibodies are present in the patient’s serum. The second is the relevance of the specificity of the autoantibodies.

(3) As the presence of alloantibodies capable of causing severe transfusion reactions can be masked by the presence of autoantibodies that react with all RBCs tested, it is essential to use techniques such as the autoabsorption technique discussed previously to determine their presence or absence. If alloantibodies are detected, blood lacking antigens to those antibodies is selected for cross matching.

(4) If the autoantibody shows clear specificit, (for example, anti-hr “(e)), blood should be selected lacking that particular antigen; there is evidence that such cells will survive much better than the patient’s own cells. If partial specificity is indicated (for example, titration technique revealing higher scores with hr"(e)-positive than hr”(e)-negative blood), the use of hr”(e)-negative blood is debatable. Limited data suggest that such blood may survive longer than the patient’s own cells. In many cases of AIHA, no specificity is obvious, the patient’s serum reacting with all normal cells tested (but perhaps marked variation is noted with cells of different donors). In cases such as this, many workers carry out compatibility tests on a large number (for example, 8 to 12) of donor units and select those units that give the weakest reactions “in vitro." Although there are no data to prove significantly better RBC survival of such comparatively weaker reactive units, it is at least esthetically pleasing for the blood transfusion laboratory personnel to issue the least incompatible units.

(5) Some investigators recommend ignoring the "specificity" of the autoantibody and, after excluding the presence of alloantibody, giving blood that is the same Rh phenotype as the patient, if feasible, in order to avoid the subsequent development of Rh alloantibodies. Such investigators emphasize that data regarding the “in vivo” significance of autoantibody specificity are scanty; however, it is also true that a significant minority of warm autoantibodies will react quite specifically with one or another of the Rh antigens, and no data exists to prove that such specificity is insignificant.

(6) Even though all blood tested is incompatible with autoantibody, transfusion may be indicated and may be a life-saving measure in cases with severe and progressive anemia. The warm auto antibody is unlikely to cause an acute hemolytic transfusion reaction and, although RBC survival may not be normal, the temporary benefit may be of great value until other therapy (for example, corticosteroids, splenectomy) is effective in producing more lasting benefit.

(7) In summary, transfusion therapy should be employed only in life-saving situations with the realization that responses are palliative and that incompatible blood is being employed. An attempt should be made to differentiate autoantibody from alloantibody in the serum. If specificity is obvious, appropriate donor blood should be selected.

NOTE: Blood should never be withheld from patients with severe life-threatening nemia because of incompatibility resulting from autoantibodies.

2-8. AUTOIMMUNE HEMOLYTIC ANEMIA ASSOCIATED WITH COLD ANTIBODIES

a. **Cold Agglutinin Syndrome.** This is the most common type of autoimmune hemolytic anemia associated with cold antibodies, comprising approximately 16 to 25 percent of immune hemolytic anemia. It can occur as an acute or chronic condition. The acute cases are often associated with Mvcoplasma pneumonias while, on rare occasions, they are associated with infectious mononucleosis, the hemolysis in these cases being transient. Patients with chronic primary cold agglutinin syndrome are often elderly, and present with chronic hemolytic anemia of mild to moderate intensity with, in cold weather, Raynaud's phenomenon, and, often, hemoglobinuria.

(1) Special technical considerations. It is preferable, if the blood is collected from the patient at 37ºC. To do this properly, blood should be collected in a warmed syringe or Vacutainer tube and immediately placed in a 37ºC water bath or thermos flask. If the samples cannot be collected at 37ºC, they should be incubated at 37ºC as soon as possible to allow autoagglutination to disperse. Fortunately, being a reversible reaction, the cold autoagglutinin will elute back into the serum from the cells. If gross autohemolysis has already occurred, fresh samples will have to be collected and the precautions previously mentioned should be taken. The serum should be separated by centrifuging the blood at 37ºC. Ideally, this means working in a 37ºC warm room or using a heated, jacketed centrifuge. If these are not available, the tubes should be centrifuged in buckets containing water at about 40ºC. The RBCs should be washed in 37ºC to 40ºC saline before they are used for grouping or direct antiglobulin tests. Following washing, it is wise to check that all autoagglutination has been dispersed.

(2) Direct antiglobulin test. Only complement is detected on the RBCs. The IgM cold autoagglutinin sensitizes the patient's RBC in the peripheral circulation where the temperature falls below 32ºC. The IgM-sensitized cells also become sensitized with complement (C3 and C4 in particular). On recirculating to 37ºC, the IgM cold antibody eluate backs off into the serum leaving the cells sensitized with complement. The C3 is acted on by C3 inactivator to leave only a fragment of C3 (C3d) on the RBCs, which is detectable by the anti-C3d in antiglobulin serums; C4d may also be present on the RBCs.

(3) Eluate from RBCs. If the RBCs have been properly collected and washed at 37ºC, no activity will be found in the eluate as only complement is present on the RBCs in "vivo" at 37ºC.

(4) Serum. lgM cold agglutinins are usually present to a titer of greater than 1,000 at 4ºC. They are usually between 2,000 and 64,000, but have been reported higher than 1,000,000. The agglutinin does not usually react above 32ºC when normal, untreated RBCs are used, but in the presence of 30 percent albumin, most of them will react up to 37ºC. Hemolytic activity against untreated RBCs can often be demonstrated at room temperature (20ºC--25ºC) and enzyme-treated RBCs are always hemolyzed in the presence of adequate complement. The lgm antibodies associated with cold chronic aggultinin syndrome are monocional proteins, almost always of the kappa light chain type. The antibodies associated with M. pneumoniae show normal kappa and lambda light chain distribution. Rare examples of lgA and lgG cold agglutinins have been described.

(5) Specificity. The most common specificity associated with cold agglutinin syndrome is anti-I; less commonly, anti-i is found, (these are usually associated with infectious mononucleosis); on rare occasions, anti-Pr, (also called anti-Sp_1), is seen. It is very difficult to determine the specificity of these cold agglutinins, unless titrations are performed. As one raises the temperature, the specificity becomes more apparent. For instance, at 4ºC an anti-I may react to a titer of 1:2,000 with adult I cells and 1:256 with i (cord) cells, but, at 25ºC, it may still react to a titer of 1:128 with adult cells and give no reaction with cord cells. If adult i cells are available, specificity is more obvious. Anti-i obviously gives the opposite results, reacting with adult i cells more strongly than i (cord) cells, and much weaker with adult cells. If all cells are equally agglutinated, a mixture of anti-I + i or anti-Pr should be suspected. Anti-Pr is easily distinguished as the antigen is destroyed by enzyme treatment, whereas both anti-I and anti-i give much higher titers against enzyme-treated cells; therefore, if both adult and cord cells give much weaker reactions after enzyme treatment, anti-Pr should be strongly suspected. To confirm the results if mixtures of antibodies are suspected, absorption and elution studies should always be carried out. These are not as simple, as when working with other blood-group systems, as no cells completely lacking the respective antigens are available.

(6) Compatibility testing.

(a) Patients suffering from cold agglutinin syndrome should not need to be transfused often, as, once diagnosed, they are told to avoid the cold; hemolysis is minimal. If the need arises, compatibility procedures should be performed with the principles that have been discussed previously kept in mind.

(b) It is advisable, if time allows, to absorb out the cold autoagglutinin using the patient's own cells. The main purpose, of this, is to ensure that no alloantibodies are present that may be masked by the cold agglutinin. Complete absorption of these high-titer cold agglutinins is very time-consuming. It helps to enzyme-treat the patient's RBCs prior to autoabsorption, but it is still a long, tedious procedure. Often, there is insufficient time to absorb sufficiently and blood may have to be issued regardless. It is a useful modification to set up room temperature

and 37ºC tests separately and in duplicate with and without the addition of albumin. The room temperature test will be strongly positive, but the 37ºC tests using saline-suspended RBCs will usually be negative if handled correctly as the antibodies usually only react up to 32ºC. If the 37ºC tests are allowed to cool, even a little, agglutination may not occur, but complement may be bound and give a positive indirect antiglobulin test (even one molecule of. the lgM anti-l can bind several hundred molecules of complement). If enough time is available, tests can be allowed to settle for 1 to 2 hours at 37ºC and read for agglutination before centrifugation where cooling may occur, leading to false-positive results. The cells are then washed, as previously described, in 37ºC to 40ºC saline at 37ºC. Once the cells are washed free of serum, they can be handled in the normal way and they no longer need to be kept at 37ºC.

(c) It should be noted that albumin, as well as enzymes, increase the strength of the cold agglutinin reaction considerably, and great care must be taken that the albumin is thoroughly warmed, before it is added to any of the tests. In many cases, one has to rely on the saline system, without the addition of albumin, in order to issue compatible blood.

b. **Paroxysmal Cold Hemoglobinuria.** Paroxysmal cold hemoglobinuria (PCA) is the rarest form of autoimmune hemolytic anemia. In the past, it was characteristically associated with syphilis, but this association seems rare nowadays. More commonly, it presents as an acute transitory form secondary to viral-like illnesses, particularly in young children. It can also occur in older people as an idiopathic chronic disease.

(1) Direct antiglobulin test. The same principles apply here as in cold-agglutinin syndrome in that the autoantibody, even though lgG, is a “cold” antibody and sensitizes RBCs in the peripheral circulation, binding complement to the cell membrane and then eluting from the cell into the serum at 37ºC. Thus, the direct antiglobulin test, if positive, is a result of sensitization with complement components only (C3d and C4d).

(2) Eluate. As in cold agglutinin syndrome, there is usually no point in making eluates from these complement-coated cells.

(3) Serum. The autoantibody has been shown to be an lgG antibody and is classically described as biphasic in that it will sensitize cells in the cold and then hemolyze them when the mixture is moved to 37ºC. This is the basis of the diagnostic test for this disease, the Donath-Landsteiner test. The antibody will often agglutinate normal cells at 4ºC, but only to low titers (for example, less than 1/64). As discussed before, it will hemolyze cells If they are sensitized in the cold (for example, melting ice) and then moved to 37ºC. The antibody usually does not sensitize cells “in vitro” above 20ºC.

(4) Specificity. The antibody has been shown to usually have specificity within the P blood-group system (for example, anti-P). That is to say, it will react with all cells, except the rare p, or P^k phenotype. Rare exceptions having anti-HI specificity have been described.

c. **Donath-Landsteiner Test.**

(1) Procedure.

(a) STEP 1. Blood from the patient is allowed to clot, undisturbed, at 37ºC and the serum separated (preferably by centrifugation at 37ºC).

(b) STEP 2. Pipette 10 volumes of patient's serum into two 10- X 76mm tubes.

(c) STEP 3. As patients with PCH may have low serum complement levels leading to false-negative results, it is recommended that tests with added complement be included. Pipette five volumes of fresh compatible normal serum as a source of complement into two 10- X 75-rnm tubes. Add five volumes of patient's serum to one tube. The tube containing complement alone acts as a negative control.

(d) STEP 4. To all four tubes add 1 volume of a 50 percent suspension of washed normal group O, P-positive RBCs.

(e) STEP 5. One of the tubes from step 2 and both tubes from step 3 are incubated, in melting ice for 1 hour.

(f) STEP 6. The other tube from step 2 is incubated in a 37ºC water bath, to act as a negative control.

(g) STEP 7. After 1 hour, the tubes in the melting ice are gently mixed and moved to 37ºC for 30 minutes.

(h) STEP 8. All tubes are gently mixed and centrifuged.

(2) Lysis of cells. Lysis visible to the naked eye indicates a positive test. The control tubes containing normal serum only, and the tube left at 37ºC (step 6) throughout the test should be negative.

(3) Alternative method. The test can also be performed by placing samples of the patient's blood into tubes prewarmed to 37ºC. One sample is left to clot at 37ºC. The other is placed immediately in melting ice and left undisturbed for 1 hour. It is then moved to 37ºC for 30 minutes, centrifuged, and inspected for hemolysis. This is a simple way of performing the test, but rather wasteful of blood.

2-9. DRUG-INDUCED "IN VIVO" RED BLOOD CELL SENSITIZATION

a. **Background.** The list of drugs capable of causing hematologic abnormalities continues to grow. Some of these abnormalities involve RBCs and some may be caused by Immune mechanisms. Many drugs seem to lead to formation of antibodies, either against the drug itself, or against intrinsic RBC antigens. Substances that act as immunogenes usually have to be of a molecular weight greater than 5,000 daltons. Drugs usually fall into the category termed by immunologists as “simple chemicals, for example, well-defined organic compounds of molecular weight under 1,000 daltons (for example, penicillin has a molecular weight of 300 daltons). It is widely accepted, that for a simple chemical to induce an immune response, it must first bind irreversibly to some tissue macromolecules (for example, protein). The conjugated proteins formed are termed hapten-protein conjugates can induce the formation of antibodies specific for the simple chemical whereas the un-conjugated simple chemical itself is incapable of inducing antibody synthesis. Although it has not been proved, it is thought that most antibodies to drugs are formed in this way. The antibodies, once formed, may cause positive direct antiglobulin tests and sometimes hemolytic anemia (see Table 2-1) by four possible mechanisms, one of which is not understood at present.

b. **Mechanisms of Erythrocyte Sensitization.**

(1) Immune-complex adsorption to RBCs.

(a) Some drugs will not bind firmly to RBCs. Even if they do combine loosely, they can easily be removed with simple washing in saline. It has been shown that drugs, such as phenacetin, and quinidine, have a high affinity for their specific antibodies, forming antigen-antibody complexes, readily in plasma. These immune complexes are capable of activating the complement cascade. This may lead to intravascular hemolysis. The RBC has a positive direct antiglobulin test (see Table 2-1, because of bound immuno-globulin-drug-complex and complement. Often, only complement is demonstrated on the RBCs. This may be explained by the fact that the immune-complex does not bind very firmly to the red cells and may dissociate from them, being then free to react with other cells. This, in turn, may explain why such a small amount of drug complex can cause so much RBC destruction.

(b) The following drugs are thought to cause a positive direct antiglobulin test and, hence, immune hemolytic anemia through this mechanism: stibophen (Fuadin), quinidine, para-aminosalicylic acid (PAS), quinine, phenacetin, insecticides (chlorinated hydrocarbons), antihistamine (Antistine), sulfonamides, isonicotinic acid hydrazide (isoniazid), chlorpromazine, amlnopyrine (Pyramidon), Dipyrone, 1-phenylalanine mustard (melphalan), Sulfonylurea (Chlorparopamide), insulin, rlfampin, and, possibly, tetracycline.

Fuadin (Stibophen)*
Quinidine
p-aminosalicylic acid PAS)
Quinine
Phenacetin
Penicillins
Chlorinated hydrocarbon insecticides (Dieidrin, Heptachior, Toxaphene)
Antihistamine (Antazole, Antistine)
Sulfonamides
Isoniazid (INH, Rifamate, Nydrazid)
Chlorpromazine (Thorazine)
Pyramidon (Aminopyrin)
Dipyrone
Methyldopa (Aldomet, Aldoril, Aldocior)
Melphalan (Alkeran)
Cephalosporins
Mefenamic acid (Ponstel)
Carbromal (Carbrital, Carbropent)
Sulfonylurea derivatives (Diabinese, Tolbutamide)
Insulin
Levodopa (L-dopa, Sinemet)
Rifamin (Rifadin, Rifamate, Rimactane)
Methadone
Tetracycline
Methysergide (Sansert)
Acetominophen
Hydrochlorothiazide
Streptomycin
Procainmide (Pronestyl, Sub-Quin)
Ibuprofen
Hydralazine (Apresoline, Hydralazise, Unipres, Serpasil)
Probenecid (Benemid)
Fenfluramine (Pondimin)
Triamterene Dyrenium)
Trimellitic anhydride
Nomifensine

*Names in parentheses are alternative names, trade names, or compounds containing the drug.

Table 2-1. Drugs that have been reported to cause positive direct antiglobulin tests and sometimes hemolytic anemia.

(c) The most common characteristics of this group of drugs in causing immune abnormalities are:

1 The patient needs to take only a small quantity of drug.

2 Acute intravascular hemolysis with hemoglobinemia and hemoglobinuria is the usual clinical presentation.

3 The antibody is often lgM.

4 Patient's RBCs are often sensitized with complement alone.

5 "In vitro" reactions (agglutination, hemolysis, and/or sensitization to antiglobulln serum) are only obvious when patient's serum, drug, and RBCs are incubated together.

(2) Drug absorbed onto RBCs.

(a) Unlike the previous group of drugs, the penicillins and cephalosporins (and possibly carbromal and methadone) bind firmly to the RBC membrane. Considerable experimental work has demonstrated that the immunogenicity is a result of its ability to react chemically with tissue proteins to form several different haptenic groups. The major haptenic determinant is the benzylpenicilloyl (BPO) group. Approximately three percent of patients receiving massive doses of intravenous penicillin will develop a positive direct antiglobulin test, and some of these will develop hemolytic anemia. The mechanism of the positive direct antiglobulin test and hemolytic anemia seems clear. The drug is absorbed to the RBCs. A immune antibody, for example, antipenicillin, is produced by the patient and will react with the penicillin on the RBCs. The end-product, therefore, is a RBC sensitized with lgG. Complement is not usually involved in this reaction, and, thus intravascular hemolysis does not usually occur. The red blood cells are destroyed extravascularly by the reticuloendothelial system, probably in the same way as red cells sensitized with lgG blood group alloantibodies (for example, Rh). If a sensitive enough technique is used, most serums can be shown to contain penicillin (BPO) antibodies. Most serums contain low-titer lgM antibodies alone; some contain lgG in addition. The high percentage of penicillin antibodies in the normal population is probably because of the continual exposure to penicillin in our modern environment. It should be noted that there is no direct correlation between the presence of penicillin hemagglutinating antibodies, and allergic reactions.

(b) The clinical and laboratory features of penicillin-induced immune hemolytic anemia are as follows:

1 Hemolysis typically develops only in patients receiving very large doses of penicillin (at least 10 million units daily for a week or more).

2 Hemolysis is subacute in onset, but may be life-threatening if the etiology is unrecognized and penicillin administration is continued.

3 A high-titer, lgG penicillin antibody is present.

4 The direct antiglobuiin test is strongly positive as a result of sensitization with lgG. (Very rarely complement may also be present.)

5 Antibody eluted from the patient's RBCs will react only against penicillin-treated RBCs.

6 Cessation of penicillin therapy is followed by complete recovery, but hemolysis of decreasing severity may persist for several weeks.

(3) The modification of RBC membrane by drugs, allowing nonimmunologic adsorption of protein.

(a) At present, the cephalosporins are the only drugs thought to react by this mechanism.

(b) The RBC membrane is modified by the drug, so that the cell now takes up proteins nonimmunologically. Cephalothin (Keflin) treated RBCs incubated in normal plasma become coated with albumin, lgG, lgA, lgM, alpha, and beta (for example, complement) globulins.

(c) As mentioned earlier, cephalothin, like penicillin, can also combine with the RBC membrane, and these cells will then react with specific anticephalothin antibodies or cross-react with antipenicillin. Any of these mechanisms may lead to a positive direct antiglobulin test. In the original reports, 40 to 75 percent of patients receiving cephalothin were found to have positive direct antiglobulin test results, but further studies found only four percent to have positive test results. There have been only two reports of hemolytic anemia resulting from cephalothin, and it is thought that these occurred through the immune mechanism involving specific antibodies to cephalothin. The principal clinical importance of positive direct antiglobulin tests caused by cephalothin is that they provide a possible source of confusion in blood bank serology or in the investigation of hemolytic disorders of other etiology.

(4) Red blood cell autoantibodies induced by drugs by unknown mechanisms.

(a) In 1966, the first positive direct antiglobulin tests and autoimmune hemolytic anemia resulting from -methyldopa (Aldomet) were described. A closely related drug, L-dopa, has also been found to cause positive direct antiglobulin tests, and two cases of hemolytic anemia resulting from this drug were described. The mechanisms involved are not understood at present. The patient forms autoantibodies

that appear to react with intrinsic RBC antigens; the serologic test results are indistinguishable from those seen in the warm autoimmune hemolytic anemias. Often, the antibody can be shown to have specificity associated with the Rh system; the serum antibody behaves independently of the presence or absence of the drug "in vitro".

(b) The clinical and laboratory characteristics of methyldopa-induced abnormalities are as follows:

1 Positive direct antiglobulin tests are found in 15 of patients receiving methyldopa.

2 The development of a positive direct antiglobulin test is dose-dependent (for example, approximately 36 percent of patients on 3 gm of drug daily develop positive direct antiglobulin tests, as compared with only 11 percent on less than 1 gm daily).

3 The direct antiglobulin test usually becomes positive after three to six months of treatment.

4 Only 0.5 to 1 percent of patients on methyldopa (Aldomet) develop hemolytic anemia.

5 The RBCs are usually sensitized with only IgG.

6 The antibodies in the serum, and eluate, are indistinguishable in the laboratory from those found in idiopathic warm autoimmune hemolytic anemia.

7 The positive direct antiglobulin test gradually becomes negative once methyldopa is stopped. This may take from one month to two years. Hematologic values usually improve within the first week or so.

(c) One other drug unrelated to methyldopa, mefenamic acid (Ponstel), has been described as a cause of immune hemolytic anemia with serologic results identical to methyldopa.

2-10. LABORATORY INVESTIGATION OP DRUG-RELATED PROBLEMS

a. The most commonly encountered problem associated with drugs is the occurrence of a positive direct antiglobulin test. A positive direct antigloblin test may of course occur with any of the drugs mentioned in this review, but methyldopa is by far the most common cause, followed by penicillin. Positive results in indirect antiglobulin tests obtained without adding drugs to the incubation mixtures are seen only in the methyldopa group as these are the only drugs that produce antibodies that react with normal untreated RBCs. The serologic evaluation of positive results of a direct or indirect antiglobulin test are basically the same as that mentioned earlier in this lesson in the investigation of autoimmune hemolytic anemia.

b. Monospecific antiglobulin serums are useful when performing the direct antlglobulin test as the immune-complex group usually presents with complement only on their RBCs, in contrast to the penicillin and methyldopa group, where, only lgG usually is present. The cells from a patient with positive results in a direct antigiobulin test as a result of cephalothln can react with some or all antiglobulin serums (for example, anti-lgG, -lgA, -lgM, -C3, -C4, -CC, -B globulins, and anti-albumin).

c. The patient's serum should be screened by the usual blood bank procedures. If antibody is detected, specificity tests should be performed and the previously mentioned facts concerning specificity-testing in warm autoimmune hemolytic anemia taken into account. If the patient's serum does not react against normal untreated RBCs, the serum should be tested against ABO-compatible RBCs in the presence of any drugs the patient has been receiving. A saturated solution of the drug should be prepared. Two useful reference books on properties, such as solubility of drugs, are the Physicians' Desk Reference, and the Merck Index. It is preferable to use several dilutions of this saturated solution, progressing to an approximate physiologic dose (for example, the average dose of drug circulating per milliliter of blood). Enzyme-treated and untreated normal RBCs should be tested. It is also wise to add fresh complement (for example, fresh compatible normal serum), to the system. The tests should be inspected for agglutination, hemolysis, and sensitization to antiglobulin sera.

d. An essential part of the investigation is to prepare an eluate from the patient's RBCs. This is particularly important in the penicillin group where, a definite diagnosis can be reached only by proving that the eluate will react with penicillin-treated cells, and yet is negative with the same cells untreated. It should be remembered that the immune complex group is often associated with red cells sensitized with complement components alone; thus, the eluates are often negative even when the drug is added to the eluate.

2-11. DETECTION OF ANTIBODIES TO PENICILLIN AND CEPHALOTHIN

a. **Preparation of Penicillin-Treated Cells.**

(1) STEP 1. Group 0 cell, (preferably fresh) are washed three times in saline.

(2) STEP 2. To one ml of packed washed cells are added 1 X 10^6 units (approximately 600 mg) of K-benzyl penicillin G dissolved in I5 ml of 0.1 M barbital buffer, pH 9.5 to 10.0.

(3) STEP 3. Incubate for 1 hour at room temperature with gentle mixing.

(4) STEP 4. Wash cells three times in saline. Slight lysis may occur during incubation, and a small "clot" may form in the RBCs, which can be removed with applicator sticks before washing cells. Once prepared, the cells may be kept in ACD, at 4ºC for up to 1 week; however, they do deteriorate slowly during this time.

b. **Preparation of Cephalothin-Treated Cells.**

(1) STEP 1. Group O cells (preferably fresh) are washed three times in saline.

(2) STEP 2. To one ml of packed, washed cells are added 400 mg cephalothin dissolved in 10 ml of pH 9.5-10.0 buffered saline (for example, one part pH 9.5-10.0 barbital buffer, plus nine parts normal saline).

(3) STEP 3. Incubate at 37ºC for two hours with gentle mixing.

(4) STEP 4. Wash cells three times in saline.

c. **Tests on Patient's Serum and Eluate.**

(1) STEP 1. If the results of a direct antiglobulin test are positive, an eluate should be prepared by a standard method (for example, heat or ether).

(2) STEP 2. The eluate and the patient's serum should be tested against normal group O cells and the same cells treated with penicillin. Usually, serial dilutions of the patient's serum are tested.

(3) STEP 3. Two volumes of eluate or serum dilutions should be incubated with 1 volume of a 2 to 5 percent suspension of penicillin-treated and untreated group O cells in saline.

(4) STEP 4. Incubate at room temperature for 15 minutes. Centrifuge and inspect for agglutination.

(5) STEP 5. Move tubes to 37ºC for 30 minutes. Centrifuge and inspect for agglutination.

(6) STEP6. Wash cells 4 times in saline.

(7) STEP 7. Add antiglobulin serum to button of washed cells. Centrifuge. Inspect for agglutination.

d. **lgM Penicillin Antibodies.** lgM penIcillin antibodies will agglutinate saline-suspended penicillin-treated cells, but not the same cells untreated. lgG penIcillin antibodies will react by the Indirect antigiobulun test against penicillin-treated cells, but not against the same cells untreated.

e. **Anitiglobulin Tests.** If indirect antiglobulin tests are used to detect cephalothin antibodies, it must be remembered that cephalothln-treated RBCs can adsorb proteins non-immunologically. Therefore, all normal serums will give positive results in indirect antiglobulin tests if incubated with cephalothin-treated cells for a long

enough period. The reaction does not usually occur once the normal protein is diluted out to more than 1/20. The amount of protein present in RBC eluates does not seem to be enough to give nonspecific results, so a positive result usually indicates the presence of antibody to cephalothin or a cross-reacting penicillin antibody.

Section II. HEMOLYTIC DISEASE OF THE NEWBORN

2-12. BACKGROUND

Hemolytic disease (HDN)of the newborn results from the immune destruction of the RBCs of the fetus and newborn by the coating of these cells with maternal lgG antibody. Individual cases vary in severity, ranging from intrauterine death to a clinically unapparent condition that can be detected only by serologic tests in a healthy baby. Close communication and cooperation between the laboratory staff, the obstetrician, and the pediatrician will greatly aid in optimal management and prevention of HDN.

2-13. CLASSIFICATION

It is convenient to classify the disease into three types based on the serologic specificity of the offending antibody. In order of severity these are:

a. **Rh (and Combinations of Rh_O).** Anti-Rh_O(D) and combinations of Rh_O with rh'(CD) or with rh"(DE).

b. **"Other".** Anti-hr'(c), anti-rh"(E), anti-KeII, and other lgG antibodies.

c. **ABO.** Anti-A, Anti-B, Anti-A, B.

2-14. MECHANISMS OF MATERNAL IMMUNIZATION

a. In hemolytic disease of the newborn (HDN) resulting from ABO incompatibility, (ABO-HDN), the lgG anti-A, anti-B, and anti-A, B are present in the mother's plasma without the requirement for prior immunization by foreign RBCs. Thus, any pregnancy, including the first, may be involved.

Abbreviations and symbols used throughout this Section are as follows:

ABO-HDN = hemolytic disease of newborn, resulting from AGO incompatibility.
HDN = hemolytic disease of the newborn.
RhIG = Rh (D) immune globulin (human).
Rh_O-HDN = hemolytic disease of newborn, resulting from anti-Rh_O(D).

b. Group O mothers, when compared with group A or B mothers, are more likely to produce lgG anti-A and anti-B. In addition, only plasma from group O persons contains lgG anti-A, B, which is often involved in ABO-HDN. ABO-HDN is almost, but not completely, restricted to group A_1 or B babies born of group O mothers.

c. In Rh_O and "other" HDN, the antibodies in the mother's plasma result from previous immunization by transfusion or pregnancy. Exposure does not always result in immunization, but production of these lgG antibodies almost never occurs without such exposure.

d. Immunization commonly results from fetomaternal hemorrhage that occurs at delivery. Small numbers of fetal RBCs possessing foreign antigens escape through the placenta and gain access to the mother's circulation, most often during the last half of pregnancy. In most instances, the small fetomaternal hemorrhage during pregnancy does not induce immunization.

NOTE: The term fetomaternal hemorrhage means the leakage of fetal RBCs into the mother's circulation. The largest fetometernal hemorrhage occurs at delivery, upon separation of the placenta. Approximately half of all women have fetal cells in their circulation in the postpartum period. The average volume of fetomaternal hemorrhage at delivery is less than 1.0 ml of whole blood; however, some women apparently become immunized by minute volumes of blood (less than 0.1ml). With volumes of Rh_O(D)-positive blood up to 0.4 ml, there is a positive correlation between the frequency of immunization and the volume of RBCs in the exposure. On rare occasions, immunization may occur during the first pregnancy by means of the transplacental passage of fetal cells. This accounts for some failures observed after prophylaxis with Rh_O(D), immune globulin, (human), (RhlG).

e. Approximately eight percen of Rh-negative women who have Rh-positive ABO-compatible babies develop detectable anti-Rh_O within six months if not protected with RhlG. An additional eight percent will develop anti-Rh_O during their next Rh-positive pregnancy. This is interpreted as a secondary response to a very small antigenic challenge with the primary immunization occurring at the prior delivery of an Rh-positive baby, even though detectable anti-Rh_O could not be demonstrated in the previous postpartum period.

f. ABO incompatibility between mother and father has the effect of protecting the mother from Rh immunization. The incidence of Rh immunization is much less following delivery of an ABO- incompatible child compared with an ABO-compatible one.

2-15. TRANSFER OF ANTIBODY TO THE FETUS

The fetus becomes passively immunized because the maternal antibody enters the fetal circulation by placental transfer. Although the exact mechanism of this transfer is unknown, it is not simple passive diffusion. The transport mechanism is very selective in that lgG is the only maternal immunoglobulin to cross the placenta. The fetus is capable of only feeble immunoglobulin synthesis; yet, at birth the fetal plasma concentration of lgG usually exceeds the maternal plasma lgG concentration. An increasing rate of transfer is evident during the last few months of pregnancy so that the

fetus attains the maternal level at about 33 weeks. At term (40 weeks), the mean IgG level of cord blood may be 20 to 30 percent higher than that of maternal blood.

2-16. CLINICAL AND LABORATORY FEATURES OF HEMOLYTIC DISEASE

a. **Pathophysiology of Hemolytic disease.** When the fetal RBCs become sensitized with maternal antibody, they are removed from the circulation by the reticuloendothelial system. The bilirubin produced from the RBC destruction crosses the placenta to the maternal circulation, where it is removed by the maternal liver. The resulting anemia causes the hematopoietic tissue of the fetus to respond by proliferating, and increasing the production of new RBCs, many of which are released prematurely into the circulation, resulting in increased numbers of reticulocytes and nucleated RBCs. The liver and spleen enlarge since much of the fetal hematopoietic tissue is located in these organs. If the immune destruction is severe, the fetal hematopoietic tissue cannot completely compensate for the loss of RBCs and the fetus becomes increasingly anemic. Severe anemia may lead to heart failure with generalized edema (hydrops fetalis), sometimes resulting in intrauterine or neonatal death. If the baby is live-born and not hydropic, the principal danger lies in the accumulation of unconjugated bilirubin. Destruction of the fetal red blood tells continues, but the maternal mechanism for excreting bilirubin is no longer present. The liver of the premature and newborn infant is unable to conjugate and excrete bilirubln effectively because of a temporary deficiency of the enzyme glucuronyritransferase. If the amount of unconjugated bilirubin exceeds the albumin-binding capacity, the unbound, unconjugated bilirubin may then diffuse into the tissue cells and result in kernicterus that is often fatal or responsible for permanent brain damage.

b. **Laboratory Features of HEMOLYTIC DISEASE.**

(1) The most useful initial index of the severity of the hemolytic process is the cord hemoglobin; however, one cannot use it as a means of subsequently excluding the need for exchange transfusion for hyperbilirubinemia. Three clinical groups may be defined as follows:

Classification	Cord Hemoglobin in gm/dl *
Mild	13
Moderate	8-13
Severe	8

*Normal cord hemoglobin is 13.6 to 19.6 gm/dl, although this may vary slightly among different laboratories.

(2) When blood is by heel prick, the hemoglobin values are often several grams per deciliter higher than in cord blood.

(3) There is an inverse relationship between the cord hemoglobin and bilirubin. That is, infants who are more severely anemic tend to have higher bilirubin levels; however, the clinical severity of HDN does not correlate as well with the bilirubin as with the hemoglobin.

(4) The strength of the positive results of a direct antiglobulin test does not correlate well with clinical severity. About half of all babies affected with HDN whose results of a direct antiglobulin test are positive do not require exchange transfusion. Nevertheless, the serologic tests on cord blood must be regarded as urgent procedures so that the pediatrician may know as soon as possible whether the baby is at risk.

(5) The major risk to the baby in the first few hours is heart failure because of severe anemia. Afterward, kernicterus or brain damage from hyperbilirubinemia is a more frequent concern. Both of these complications may be prevented by exchange transfusion. Sequential accurate hemoglobin and serum bilirubin determinations are of major importance in the decision regarding when, and if, exchange transfusion is indicated.

c. **Late Anemia.** Occasionally, babies affected with HDN develop a significant anemia at 3 to 5 weeks of age caused by their reduced hematopoietic activity and continued hemolysis. Since the diagnosis of HDN may not be suspected, all anemic infants in the first 3 months of life should have laboratory investigations carried out to evaluate this possibility (see para 2-18, this section, on Laboratory investigation of HDN during the neonatal period).

d. **ABO-HEMOLYTIC DISEASE OF NEWBORN.** Hemolytic disease caused by ABO incompatibility has special features. It is important to separate ABO sensitization from clinically significant ABO hemolytic disease. Anemia is often absent or minimal in this mild hemolytic process. Jaundice may occur in the first 24 to 48 hours of life (later than in Rh disease) and this usually first suggests the diagnosis. The hemolytic process may be evaluated by examination of blood smears for an increased erythrocyte sphering, reticulocytosis and increased numbers of nucleated RBCs. No tests are available for the accurate prenatal prediction of ABC hemolytic disease. Since the disease is usually mild and exchange transfusion is infrequently necessary, early delivery is never indicated.

2-17. PRENATAL STUDIES

a. Red Blood Cell Typing and Serologic Studies.

(1) The objective of prenatal immunohematologic studies is to identify those women at risk of having babies affected with HDN. Once identified, these women can be followed in order to estimate the degree of involvement, to determine the optimal time for delivery, to notify the blood bank before delivery in order that blood be available for possible exchange transfusion, and to alert the pediatrician for the arrival of a possibly affected newborn. An obstetrical and transfusion history should be included as part of the first obstetrical visit. Relevant tests to be requested on the first visit include:

(a) ABO grouping, 2 Rh typings, and 3 tests for unexpected antibodies.

(b) If the woman is Rh-negative, her blood should be routinely checked for the Rh_O variant (D^U). When using a suitable serum, the result of this test should be read microscopically if macroscopically negative and the results recorded to indicate a microscopic reading.

(2) In Rh-negative women whose results in an original antibody-detection test were negative, the test should be repeated at approximately 32 weeks. If the results of this test are negative, no further prenatal serologic tests need be made. All positive antibody-detection tests require identification of the antibody. Antibodies which are not formed as a result of known RBC sensitization (for example, anti-Lewis, anti-lH, anti-H, and anti-I) are relatively common during pregnancy, but do not cross the placenta. Treatment of the serum with two-mercaptoethanol or dithiothreitol will aid in distinguishing lgM from lgG antibodies. All significant antibodies should be titered and scored at least monthly or until amniocentesis is begun. A change in titer of more than two tubes (over fourfold) or a score change of more than ten is significant. Changes in titer of one tube or score changes of less than five are not significant. Score changes of 5 to 10 and titer changes of two tubes are equivocal. When prenatal testing reveals an unexpected antibody, freeze the serum (-30ºC) for comparison with subsequent samples from the patient.

(3) The principal value of antibody titration is to identify those women who are candidates for amniocentesis. A rising titer in the first affected pregnancy indicates that the baby probably will be affected with HDN. Serial titers in subsequent pregnancies may have some prognostic value. Antibody titration is generally not helpful in the prenatal management of ABO hemolytic disease.

(4) In Rh hemolytic disease, Rh phenotyping of the child's father can be helpful in predicting the outcome of future pregnancies. In other types of HDN, the father's RBCs can be tested to determine if he is homozygous or heterozygous for the gene producing the immunizing antigen.

b. **Amniotic Fluid Examination.**

(1) Examination of the amniotic fluid for bilirubin like pigment is the best available method for evaluating the degree of hemolysis occurring in the infant and the infant's general condition. There are two indications for performing amniocentesis:

(a) Antiglobulin titer of 1:32 or higher for an unexpected antibody known to be capable of causing HDN. (This minimal critical titer may vary slightly in different laboratories because of differences in technique.)

(b) A history of a previously affected baby with Rh_O or other HDN, regardless of the maternal antibody titer.

(2) The initial amniocentesis is usually done at about 26 weeks, but may be performed earlier if there is a past history of a baby with HDN. Amniocentesis should not be performed without clear indications since the procedure may cause infection, provoke more severe sensitization if the needle allows the escape of fetal blood into the mother's circulation, or may precipitate labor or fetal death. Amniocentesis is never indicated in ABO-HDN.

(3) Normal amniotic fluid is colorless. The fluid becomes increasingly yellow with increasing severity of fetal hemolytic disease. When the amniotic fluid is obtained, it can be centrifuged and the small number of RBCs present can be typed for ABO and Rh. If a KIeihauer blood smear is performed on these RBCs, it may be possible to tell whether the cells are adult or fetal, and thus the infant's blood type may be predicted.

(4) Interpretation of the amniotic fluid analysis depends on sequential studies repeated at 2-week intervals, or closer if indicated. A spectral absorbance curve from 350 to 750 nm is made of the amniotic fluid. A line is projected from 375 to 525 nm and the change in absorbance seen at 450 nm (Δ0D450) is plotted on the Liley graph according to the appropriate week of gestation. Trends are much more valuable than isolated points. If the Δ0D450 is in the low zone or falls into the low zone as the pregnancy progresses, the baby will be unaffected or have mild disease. A middle zone pattern should suggest that the baby will probably be moderately affected, and one should prepare for an exchange transfusion after an early delivery. With Δ0D450 values above 0.3 in the upper zone or a trend from the middle zone to the upper zone, fetal death is imminent. If the gestation is at least 32 to 34 weeks, the pregnancy is usually interrupted to deliver the baby. Before 32 to 34 weeks, the risk to neonatal death resulting from prematurity is very high, so a intrauterine transfusion may be indicated. A determination of the lecithin/sphingomyelin ratio on the amniotic fluid may be valuable in predicting fetal lung maturity, and thus the ability of the fetus to survive early delivery.

2-18. LABORATORY INVESTIGATION OF HEMOLYTIC DISEASE OF NEWBORNS DURING THE NEONATAL PERIOD

a. Unsuspected Hemolytic Disease of The Newborn.

(1) A clotted sample of cord blood (preferably collected by needle and aspiration) should be obtained on every newborn. This tube should be labeled in the delivery suite with the mother's name, baby's identification (for example, Baby Boy Smith or baby's name), hospital number, and date. Such samples should be sent to the blood bank, and stored in the refrigerator for at least 7 days.

(2) This blood is then readily available for testing if the newborn develops signs and symptoms suggestive of HDN. This blood sample also can be used, if it is desired, to test certain special groups of patients, such as all babies of group O mothers, Rh-negative mothers, or mothers who have delivered a baby affected with HDN. If these patients are to be studied, the following tests are recommended: (1) ABO group, (2) Rh_O(D) type, and D^U, if Rh-negative, and (3) direct antiglobulin test.

b. **Laboratory Investigation of Suspected Hemolytic Disease of Newborns.**

(1) A clotted sample of cord blood, and maternal blood should be obtained. The mother's blood should be tested for: (1) ABO group, (2) Rh_O(D) type, and D^U if Rh_O(D) negative, (3) unexpected RBC antibody, and (4) identification of antibody if results of detection test are positive. The infant's blood should undergo the following tests: (1) ABO group, (2) Rh_O(D) type and D^U if Rh_O(D) is negative, (3) direct antiglobulin test, (4) elution if results of direct antiglobulin test are positive, and (5) identification of antibody in the eluate.

(2) Because alloantibodies present in cord serum are IgG antibodies of maternal origin, the ABO group of the baby is based entirely on cell grouping. If the newborn has received repeated intrauterine transfusions of group O Rh-negative, packed, RBCs, spurious results may be found in ABO grouping, Rh-typing, and in the direct antiglobulin test.

(3) It may be particularly difficult to perform Rh-typing accurately in patients with HDN. Several additional phenomenon may be present in patients with HDN and cause false reactions in Rh-typing. False-positive reactions may occur because of: (1) the presence of Wharton's jelly in incompletely washed RBC samples, and (2) coating of Rh-negative red cells by an antibody other than anti Rh_O(D), for example, anti-hr'(c).

(4) Enhancement of agglutination of these situations may be caused by potentiators added to the slide or rapid tube test anti-Rh_O(D) serum. The use of an albumin control or saline anti-Rh_O(D) serum will aid in detecting this anomaly.

(5) False negative or very weak positive Rh-typing is occasionally encountered when the newborn is Rh-positive and the red blood cells saturated with maternal anti-Rh_O(D) so that all of the Rh_O(D) antigen sites are blocked ("blocked D"). This condition should be suspected when the mother is Rh-negative and the results of the baby's direct antiglobulin test are strongly positive.

(6) The results of a direct antiglobulin test are usually strongly positive in Rh_O and "other" HDN, while they are usually weakly positive or negative in ABO-HDN. Identification of antibody in the eluate of cord cells should be done whenever the results of the direct antiglobulin test are positive. If the mother's serum is ABO-incompatible with the baby's cells and doesn't have any unexpected antibodies, the eluate should be tested against adult A_1, B, and O cells by saline and antiglobulin techniques. The procedure must be used to diagnose ABO-HDN. If the results of the direct antiglobulin test are positive and an antibody cannot be demonstrated in the mother's serum or in an eluate from the cord blood, suspect HDN resulting from a private or low-incidence factor. This can be confirmed by testing the mother's serum with the father's RBCs, if mother and father are ABO-compatible. If they are ABO-incompatible, the mother's serum should be repeatedly absorbed at 4ºC and 37ºC utilizing a random cell of the

appropriate ABO group to remove the incompatible anti A, or anti B. When all this antibody activity has been removed, her serum may then be tested against the father's RBCs.

(7) When the mother is known to be immunized to any RBC antigen, cord blood should be tested for: (1) hemoglobin and hematocrit, (2) serum bilirubin, (3) blood smear evaluation, and (4) blood bank studies described in para 2-18b(1) of this section.

2-19. TREATMENT OF HEMOLYTIC DISEASE OF THE NEWBORN

a. **Prenatal Treatment-Intrauterine Transfusion.**

(1) Intrauterine transfusion carries a high risk of fetal mortality and, therefore, must be performed only, after careful evaluation of the problem by trained and experienced physicians. Red blood cells less than 5 days old may be used for intrauterine transfusion. Many physicians prefer to use frozen deglycerolized red cells because the risk of hepatitis and graft-vs-host disease may be reduced. The red cells should be group O and compatible with the mother's serum. Once initiated, such transfusions are usually repeated every 2 weeks until delivery.

(2) At birth, babies with Rh_OHDN who have had intrauterine transfusions often type as Rh-negative (or weakly mixed field positive) and the results of a direct antiglobulin test are negative (or weakly mixed field positive). These observations are a result of the fact that, at birth, over 90 percent of the baby's blood may be that of the donor. In rare patients with unusual high-frequency antibodies, it may be desirable to collect blood from the mother and store the RBCs in the frozen state for either Intrauterine or exchange transfusion.

b. **Neonatal Treatment-Exchange Transfusion.**

(1) <u>Objectives of exchange transfusion:</u>

(a) To lower the serum bilirubin concentration in order to prevent kernicterus.

(b) To remove the baby's RBCs that have been coated with antibody, and would be more rapidly destroyed.

(c) To provide substitute compatible RBCs with adequate oxygen-carrying capacity.

(d) To reduce the amount of incompatible antibody in the baby.

(2) Selection of blood for exchange transfusion.

(a) Donor blood selected for exchange transfusion should:

1 Lack the RBC antigens corresponding to the maternal antibodies.

2 Be crossmatched with the mother's serum.

3 Be less than 5 days old.

(b) For ABO-HDN, group O RBCs of the same Rh type as the baby should be used for exchange transfusion. In order to avoid transfusing the anti-A, and anti-B in the plasma, group O RBCs, either as RBCs or frozen deglycerolized, can be used in combination with compatible plasma (for example, group AB). If group O whole blood is used for the exchange transfusion, the whole blood must be free of excessive hemolytic anti-A and anti-B activity.

(c) In $Rh_{O,}$ and "other" HDN, it is desirable to perform the exchange transfusion with blood of the infant's own ABO group, when the mother's serum is ABO-compatible. If the infant's condition is so critical that exchange transfusion cannot be delayed and the donor unit is to be prepared prior to delivery, for Rh_OHDN, select a group O Rh_O-negative unit that lacks excessive hemolytic activity. For "other" HDN when the donor unit is prepared prior to delivery, select a group O Rh_O-negative unit which lacks the corresponding RBC antigen and which is low in hemolytic anti-A or anti-B activity. In either RH_O-HDN or "other" HDN, it may be helpful to prepare a donor unit of the mother's ABO group simultaneously. This may make it possible to use group-specific blood if the exchange transfusion can be delayed until the infant's own ABO group is determined.

(d) Select donor blood of the baby's ABO group if it is compatible with the mother's serum (if not, group O is used), if the request for the donor unit is received after the baby has been blood-typed There is no advantage in using Rh-negative donor blood for an Rh-positive baby unless the hemolytic disease is caused by anti-Rh_O(D).

e) When the decision is made as to the proper ABO and Rh type of donor blood to use, select the freshest processed unit of this type for the compatibility tests. The maximum age for this unit should be less than 5 days from collection. This requirement is based on the three following characteristics important to the baby:

1 Maximum RBC viability to minimize the extra pigment, and the potassium loads released from nonviable RBCs.

2 Maximum two, three-DPG levels to ensure immediate good hemoglobin function.

3 Plasma electrolyte concentration, within limits, which are tolerable for infants.

(f) If the clinical situation is critical, slightly older blood may be used. CPD blood is suitable for use in exchange transfusions, but some prefer heparinized blood.

(3) Compatibility testing for exchange transfusion.

(a) The compatibility tests should be performed using the mother's serum and the donor's red cells. The mother's serum is the specimen of choice since the offending antibody is present to a higher titer in her serum than in the infant's serum. If the mother's blood cannot be obtained, the compatibility test is performed using an eluate of the cord cells or, if time is critical, the baby's serum. It is important not to cause needless delay by waiting for a maternal blood sample that may require some time to obtain.

(b) In repeated exchange transfusion, subsequent units should be the same blood group, and type as the first unit. After each exchange, a sample of blood should be drawn from the infant and used in compatibility testing for future exchange transfusion. In addition, it is advisable to use samples of maternal serum.

(c) A common problem is the presence in the maternal serum of anti-Lewis, IH, I, or H, as previously described, in addition to the IgG antibody responsible for the hemolytic disease. Such cold IgM antibodies may delay locating completely compatible blood unless they are ignored. When present, donor units incompatible with the maternal serum may be used for the exchange transfusion provided they lack the antigen that corresponds to the maternal IgG antibody. Alternatively, one may use maternal serum for the compatibility test after treatment with two-mercaptoethanot or dithiothreitol.

(d) Compatibility tests after delivery are best performed using the cord cell eluate, when this problem exists.

(4) Exchange transfusion in patients with an antibody to a high-frequency antigen.

(a) Rarely, the mother's serum may contain an antibody to a high-incidence factor and no compatible blood is available. Recognition and identification of this problem during pregnancy will allow the blood bank time to work with the AABB Rare Donor File to locate compatible donors and to test the siblings of the mother for compatibility and suitability. If this problem is not recognized until after delivery and the need for donor blood is urgent, three choices are open.

1 If available, test the mother's siblings for compatibility and suitability.

2 Collect one unit of blood from the mother. Centrifuge the whole blood and remove as much plasma as possible. Resuspend her RBCs in group AB plasma.

3 Use fresh donor blood that possesses the antigen and is incompatible.

(b) Incompatible blood has been used with success in many cases, and one should not hesitate to use it if no compatible blood is available, the mother's donor status is questionable, and the clinical situation is urgent.

2-20. Rh_O(D) IMMUNE GLOBULIN (HUMAN)-RhIG

a. Experience has shown that the routine administration of RhIG to nonimmunized Rh_O-mothers who deliver Rh-positive babies is an effective means of preventing Rh-alloimmunization. Candidates for this prophylaxis are mothers who are Rh-negative and D^U-negative, have no detectable anti-Rh_O(D) antibody, and have an Rh-positive, or D^U-positive newborn.

b. Since Rh antigens have been found on fetal RBCs quite early during gestation, all Rh-negative women who have abortions are candidates, unless the father or fetus is known to be Rh-negative.

c. The following women are not RhIG candidates:

(1) Rh_O-negative women who deliver Rh-negative babies.

(2) Rh_O-negative women whose serum contains anti-Rh_O(D), Rh_O(D) negative women who deliver Rh-positive babies and whose serum contains other unexpected antibodies (for example, anti-KeII) are candidates.

(3) Rh_O-positive or D^U-positive women.

d. Administration of RhIG is indicated, but sometimes inadvertantly omitted after several common events such as amniocentesis, antepartum hemorrhage, or ectopic pregnancy.

(1) If pregnancy in an Rh-negative woman terminates before 13-weeks gestation, a dose of 50 ug is adequate to cover the small fetal blood volume during the first trimester. From 13 weeks until term, the standard 300 ug dose should be given.

(2) Since fetomaternal hemorrhage may accompany amniocentesis, the procedure can cause Rh immunization. The Rh-negative woman who has amniocentesis at 16 to 18 weeks for genetic analysis should receive a 300 ug dose of PhIG. A second dose should be given 12 weeks later, or at 28 weeks gestation, and a third dose given after delivery if the baby is Rh-positive. Amniocentesis performed in the second or third trimester of pregnancy on the non-immunized Rh-negative woman

should be followed by the injection of 300 ug RhIG. If a subsequent amniocentesis is done more than 21 days later, an additional injection of 300 ug RhIG should be given.

e. RhIG is supplied as sterile, clear, and injectable for intramuscular administration into the mother within 72 hours after delivery if she meets the above criteria. It is a highly concentrated solution of IgG anti-Rh_O(D) (about 300 ug of anti-Rh_O(D) globulin) derived from human plasma. RhIG, like other immune serum globulin preparations, does not transmit hepatitis.

f. If the newborn is known to be Rh_O(D)-positive, and the blood bank receives a request for RhIG, the following procedures should be done before administering the RhIG to the mother:

(1) ABO grouping of mother.

(2) Rh_O(D)-typing of mother, including D^U test read microscopically.

(3) Testing of mother's serum for unexpected antibodies.

(4) A compatibility test between the mother's red blood cells and the sample of diluted RhIG included with each vial of RhIG is optional.

g. Anti-Rh_O(D) antibody can be detected In the mother's serum 12 to 60 hours after injection, and may continue to be detected for as long as 5 months. If anti-Rh_O(D) is present 6 months after delivery, it can be assumed that this represents active immunization, and failure of the RhIG to block alloimmunization. Such failures are infrequent, and may be caused by undetected Rh_O sensitization, that existed prior to the administration of RhIG. RhIG does not reverse Rh sensitization, but rather prevents its occurrence. Early in Rh_O alloimmunization, the antibody may not be detectable by standard antibody-screening procedures.

h. The amount of RhIG supplied in one container is generally sufficient, if the fetomaternal blood is 30 ml or less. The entire contents of the container should be injected, since the volume of Rh-positive fetal cells that enters the maternal circulation is unknown.

2-21. MASSIVE FETOMATERNAL HEMORRHAGE

a. When a massive fetomaternal hemorrhage occurs (greater than 30 ml of whole blood), one container of RhIG is an insufficient dose to suppress alloimmunization from this larger immunogenic challenge. Recognition of the massive fetomaternal hemorrhage is of great importance. A careful microscopic examination should be made using the mother's postpartum specimen to detect a massive hemorrhage. The most practical method for its detection is the search of a mixed field pattern of micro-agglutinates among a background of un-agglutinated cells, in the microscopic reading, of the D^U test in the mother. The material supplied by the

manufacturers for compatibility-testing is less sensitive than the reagent anti-Rh_O(D). The microagglutinates consist of Rh-positive fetal cells coated with reagent anti-Rh_O(D) reacting with the antiglobulin serum.

b. Agglutination in the microscopic phase of the D^U test may not indicate a massive fetomaternal hemorrhage, but may be caused by:

(1) The patient being a weak D^U.

(2) The reagent anti-Rh_O(D) slide or rapid tube test serum not being standardized for close microscopic reading in the antiglobulin test.

(3) "Contamination" of the anti-Rh_O(D) reagent serum with a second weaker antibody that is reacting with the patient's RBCs.

c. Confirmation of the hemorrhage is established by the acid-elution test of Kliehauer-Betke. This test is performed on a postpartum blood smear of the mother.

d. If the diagnosis of a massive fetomaternal hemorrhage is confirmed, the volume of the fetomaternal hemorrhage must be determined, in order to calculate the number of vials of RhIG, to administer. Volume estimates are probably best determined from the acid-elution test. One vial of RhIG is sufficient for 15 ml packed cells (30 ml of fetal whole blood). Since the accuracy of the acid-elution test is poor (half to twice the actual volume of the hemorrhage), it is preferable to administer a slight excess of RhIG. A simple method described by Kliehauer for calculating the volume of the fetomaternal hemorrhage gives an estimate that is equal in accuracy to more complicated formulas. The volume of the fetomaternal hemorrhage can be calculated by multiplying the percentage of fetal cells times 50. Since each dose of RhIG protects against sensitization from approximately 30 ml of fetal blood, the volume of the fetomaternal hemorrhage is divided by 30 and multiplied by 2. The two is a correction factor because the Klliehauer estimate of fetomaternal hemorrhage may be as little as one half the actual volume of the hemorrhage.

Example:

Counting of 2,000 cells in acid elution smear reveals 1.2 percent fetal cells

$1.2 \times 50 = 60$ ml fetomaternal hemorrhage

$\frac{60}{30} \times 2$ $\quad 2 \times 2 = 4$ vial of RhIG required

e. When multiple vials of RhIG are to be administered to the mother, base line

studies of hemoglobin, and bilirubin should be obtained. Several vials of RhIG can be pooled in one syringe for a single injection. Not more than 5 ml should be injected at one time into each buttock. If more than ten doses are required, the injections should be spaced over the 72-hour period; however, the optimum time sequence for these injections has not been established. Failure of this method to prevent Rh alloimmunization usually means that the massive fetomaternal hemorrhage was chronic.

2-22. MATERNAL ALLOIMMUNIZATION TO PLATELETS, NEUTROPHILS, AND PROTEIN ALL O TYPES

a. Neonatal thrombocytopenic purpose is an infrequent transient condition that occurs by a mechanism analogous to the fetal RBC destruction in HDN. It is usually caused by the platelet IgG alloantibodies anti-P^{1A} and ant -P^{1E2}.

b. Leukocyte lgG alloantibodies are common in the serums of pregnant women, but they rarely cause problems in the newborn.

c. Although maternal alloimmunization to fetal Gm factors has been documented, no deleterious effects are evident in the fetus or newborn. Gm antibodies are primarily in the lgM class and, thus, would not cross the placenta.

Continue with Exercises

EXERCISES, LESSON 2

INSTRUCTIONS: Answer the following exercises by marking the lettered response that best answers the exercise, by completing the incomplete statement, by selecting true or false, or by writing the answer in the space provided at the end of the exercise.

After you have completed all the exercises, turn to “Solutions to Exercises at the end of the lesson and check your answers. For each exercise answered incorrectly, reread the material referenced with the solution.

1. The survival of red cells is shortened when hemolysis results from an immune reaction of a patient with immune hemolytic anemia.

   a. True

   b. False.

2. About two-thirds of the immune hemolytic anemia cases are associated with:

   a. Drugs.

   b. Warm antibodies.

   c. Cold agglutinins.

   d. Paroxysmal cold hemoglobinuria.

3. Clotted blood and blood in EDTA are collected to provide _____ and _____ respectively for general serologic investigations of AIHA.

   a. Serum; cells.

   b. Cells; plasma.

   c. Antigens; antibodies.

   d. Cold sample; warm sample.

4. If powerful cold autoantibodies are suspected, blood samples for investigation of AIHA should be kept at:

   a. 4ºC.

   b. 37ºC.

   c. Room temperature.

5. Red blood cells are tested first with _____ antiglobulin reagent and then with _____ antiglobulin serum for the serologic investigation of AIHA.

   a. Cold; warm.

   b. Active; inactive.

   c. Broad-spectrum; monospecific.

   d. Monospecific; broad-spectrum.

6. What are the autoantibodies directed against in most cases of the warm antibody of AIHA?

   a. ABO antigens.

   b. Ii antigens.

   c. Lewis antigens.

   d. Rh complex.

7. Why is it important not to expose the patient to Rh antigens, especially D, when testing for specificity of the autoantibody?

   a. WAIHA are complex.

   b. Their own cells lack that antigen.

   c. Often there are low serum complement levels.

   d. There are difficulties in autoagglutination groups.

8. Which type of autoantibodies are predominant in the warm antibody of AIHA?

    a. lgA.

    b. lgD.

    c. lgG.

    d. lgM.

9. Blood transfusions are usually indicated in AIHA.

    a. True.

    b. False.

10. Which type of autoantibodies is predominant in the cold antibody of AIHA?

    a. lgA.

    b. lgD.

    c. lgG.

    d. lgM.

11. Hemolytic anemia may be developed in patients (less than one percent) during long-term therapy with:

    a. Phenacetin.

    b. Penicillin.

    c. Methyldopa (Aldomet).

    d. Sodium cephalothin (Keflin).

12. Hemolytic Disease of Newborns destroys RBCs of the fetus or newborn by maternal IgG antibodi

    a. True.

    b. False.

13. What maternal antibody may be involved in ABO-HDN?

    a. Anti-A.

    b. Anti-B.

    c. Anti-A, B.

    d. All of the above.

14. What maternal antibody is involved in Rh_O-HDN?

    a. Anti-A.

    b. Anti-B.

    c. Anti-A1, B1.

    d. Anti-Rh_O(D).

15. ABO-HDN occurs mostly in babies of group A_1 or B with mothers of group:

    a. A.

    b. B.

    c. AB.

    d. O.

16. Antibodies are responsible for Rh_O-HDN and "other" HDN usually due to previous pregnancy or transfusion.

    a. True.

    b. False.

17. What maternal antibody is able to cross the placenta to the fetus?

    a. lgM.

    b. lgG.

18. What is a major risk to a baby with HDN?

    a. Heart failure and brain damage.

    b. Liver damage and loss of sight.

    c. Bone marrow damage and deformity.

    d. Growth inhibition and tooth discoloration.

19. What is the most useful initial index to the severity of the hemolytic process in HDN?

    a. Cord hemoglobin.

    b. Serum bilirubin level.

    c. Blood smear evaluation.

    d. Erythrocyte sedimentation rate.

20. What do almost all babies with a positive direct antiglobulin test require?

    a. lgH.

    b. ACT.

    c. Nothing.

    d. Exchange transfusion.

21. Which of the following is NOT a routine test for the first prenatal visit?

    a. ABO grouping.

    b. Direct antiglobulin test.

    c. Screening for unexpected antibodies.

    d. Rh typing (and test for D^U if Rh-negative).

22. Antibody identification is necessary if an antibody screening test is positive during pregnancy.

    a. True.

    b. False.

23. If a titer of an antibody increases by two tubes, and the score increases by 10, the change is:

    a. Equivocal.

    b. Significant.

    c. Insignificant.

24. Amniocentesis is NEVER indicated in:

    a. Rh_O-HDN.

    b. ABO-HDN.

    c. "Other" HDN.

25. A mother who is O negative with a past history of HDN gives birth. Identify the test which would NOT routinely be performed on the newborn.

    a. ABO grouping.

    b. Direct antiglobulin test.

    c. Screening for unexpected antibodies.

    d. Rh typing (and test for D^U if Rh-negative).

26. Why should the eluate from the red cells be tested if the direct antiglobulin test of an infant's blood is positive?

    a. Determine the titer.

    b. Identify the antigen.

    c. Identify the antibody.

    d. Screen for antibodies.

27. Cord blood should be obtained for _____ when the mother carries antibodies against a red cell antigen.

    a. Blood bank studies.

    b. Serum bilirubin level.

    c. Blood smear evaluation.

    d. Hemoglobin level and hematocrit.

    e. All of the above.

28. The maximum desirable age of donor blood for exchange transfusion in HDN is less than:

    a. 5 days.

    b. 17 days.

    c. 21 days.

    d. 28 days.

29. A donor unit should be group _____ , Rh-_____ , and without excessive hemolytic activity when it is selected prior to delivery for exchange transfusion in Rh_O-HDN.

    a. AB; negative.

    b. AB; positive.

    c. O; negative.

    d. O; positive.

30. RhIG prophylaxis is indicated If a woman:

    a. Is Rh-negative.

    b. Is D^U-negative.

    c. Has no detectable anti-Rh_O(D).

    d. Has an Rh-positive or D^U -positive newborn (or aborted fetus not shown to be Rh-positive.

    e. All of the above.

31. What is the preliminary indication of a newborn when a microscopic D^U test of the mother reveals a mixed field pattern of microagglutinates among a background of unagglutinated cells?

    a. AIHA.

    b. Kernicterus.

    c. Viral hepatitis.

    d. Massive fetomaternal hemorrhage.

Check Your Answers on Next Page

SOLUTIONS TO EXERCISES: LESSON 2

1. a (para 2-2a)
2. b (para 2-2c)
3. a (para 2-3a)
4. b (para 2-3a)
5 c (para 2-3b(1) Questions 1 and 2)
6. d (para 2-3c(2)(c))
7. b (para 2-4)
8. c (para 2-7b)
9. b (para 2-7f(1))
10. d (para 2-8a(2))
11. c (para 2-9b(4)(b)4)
12. a (para 2-12)
13. d (para 2-13c)
14. d (para 2-13a)
15. d (para 2-14b)
16. a (para 2-14c)
17. b (para 2-15)
18. a (para 2-16a)
19. a (para 2-16b(1))
20. d (para 2-16b(4))
21. b (para 2-17a(1))

22. a (para 2-17a(2))

23. a (para 2-17a(2))

24. b (para 2-17b(2))

25. a (para 2-18a(2))

26. c (para 2-18b(6))

27. e (para 2-18b(7))

28. a (para 2-19b(2)(e))

29. c (para 2-19b(2)(c))

30. e (para 2-20a)

31. d (para 2-21a)

End of Lesson 2

LESSON ASSIGNMENT

LESSON 3	Blood Transfusion
TEXT ASSIGNMENT	Paragraph 3-1 through 3-18.
LESSON OBJECTIVES	After completing this lesson, you should be able to:

3-1. Identify the indications, contraindications, reactions, advantages, disadvantages, and administrative guides for red cell products, platelets, coagulation factors, and plasma substitutes.

3-2. Identify the common causes of transfusion reactions and methods of investigation.

3-3. Identify the guidelines for prevention of hepatitis transmission.

SUGGESTION After completing the assignment, complete the exercises at the end of this lesson. These exercises will help you to achieve the lesson objectives.

LESSON 3

BLOOD TRANSFUSION

Section I. BLOOD TRANSFUSION PRACTICE

3-1. BACKGROUND

The development of methods for preserving RBCs and other blood components has made transfusion readily available. Despite the relative ease of transfusion, it may have serious complications and should be undertaken only after considering the etiology and course of the patient's disease and clinical condition. If transfusion therapy is indicated, the specific blood traction that is lacking should be identified, and a specific blood component used to replace that deficit. Usually the patient is best served when specific blood component therapy is utilized.

3-2. TRANSFUSION OF BLOOD PRODUCTS CONTAINING RED BLOOD CELLS

a. **Indications for Transfusion of Red Blood Cells (Human) and Whole Blood (Human).**

(1) The most common reasons for transfusion are replacement of RBCs for oxygen-carrying capacity or restoration of blood volume. In deciding whether a patient requires RBC transfusion, the clinical condition of the patient is of primary importance. The amount of blood loss that can be tolerated without replacement depends upon the condition of the patient. If blood loss has been acute, the patient may have normal or nearly normal hemoglobin, but may, nevertheless, require transfusion for the restoration of blood volume. If blood loss is judged sufficient to require transfusion, it is not necessary to wait until symptoms such as pallor, diaphoresis, tachycardia, or hypotenslon develop. On the other hand, transfusion should not be initiated too rapidly because it seems clear that, in most normal ”patients," the loss of approximately 1,000 ml can be replaced by colloid or crystalloid solutions alone.

(2) When anemia has developed over a long period of time, the patient adjusts to lower hemoglobin levels and may not require transfusion despite very low hemoglobin levels. The condition of the patient is of primary importance, not the laboratory values. There is no evidence that it is necessary to transfuse a patient to a "normal” hemoglobin prior to surgery, nor is there any specific hemoglobin value above that patients feel better or wound healing is improved. In patients with chronic anemia, attempts should be made to diagnose and treat the anemia. Transfusion should be used only as a last resort since it may suppress erythropoiesis.

b. **Red Cell Products.**

(1) Whole blood. Whole blood (WB) is the product of choice for acute massive blood loss in many hospitals. Others prefer to use colloid or crystalloid solutions followed by RBC. In acute massive blood loss, the blood volume deficit is as important as the loss of blood cells and transfusion therapy must replace this volume deficit. Whole blood may be required for certain special circumstances, such as exchange transfusion; however, exchange transfusion can be performed with RBC and either albumin or fresh frozen plasma.

(2) Red Blood Cells.

(a) Red blood cells are the product of choice to restore or maintain oxygen-carrying capacity. Patients who have chronic anemia, congestive heart failure, or are elderly or debilitated tolerate poorly, rapid changes in blood volume. Transfusing RBC increases oxygen-carrying capacity with minimal expansion of blood volume. Nonhemolytic transfusion reactions occur less frequently after transfusion of RBCs than after WB, probably because most platelets, granulocytes, and plasma are removed.

(b) The use of RBC in surgery is more controversial. Some surgeons and anesthesiologists feel that the blood loss that occurs during surgery is acute and should be replaced with WB. Other surgeons, anesthesiologists, and most immunohematologists believe that surgical blood loss usually occurs under controlled conditions can be replaced with RBCs and saline. Investigations have shown no increased morbidity or mortality from the use of RBC, and this practice is common in many hospitals. In addition a number of studies have shown that 1,000 to 1,200 ml of operative blood loss can be replaced by electrolyte and/or colloid solutions without the use of RBCs. Thus, the loss of two units of blood during "routine" surgery could be replaced with RBCs. The use of RBCs allows platelet concentrate and plasma products to be produced from the same unit of blood. Because this is a much more efficient use of the original unit of blood, RBCs are always preferred, unless there is a specific indication for the use of WB.

(3) Fresh whole blood.

(a) An order for fresh blood must be interpreted by the blood bank staff as a cry for consultative help since the uses of fresh blood are often misunderstood by the patient-care physician. Some patients may have a RBC or blood volume deficit combined with depletion of functional platelets and/or decreased levels of coagulation Factors V and VIII; however, it is difficult to replace these deficiencies using fresh WB because of the volume required. Patients who are thrombocytopenic usually require more platelets than are present in one unit of blood. Thus, platelet concentrations should be used. Deficiency of coagulation factors is usually better managed with cryoprecipitate, fresh-frozen plasma, or concentrated coagulation factors than with fresh WB.

(b) As pretransfusion testing of donor blood has become more complex, the time required to make fresh blood available has increased. It is preferable to maintain stock supplies of cryoprecipitate, fresh-frozen plasma, or concentrated coagulation so that patients requiring replacement of platelets, coagulation factors, and RBCs can be treated with appropriate mixtures of these components. Usually, these previously tested components can be made available to patients much more rapidly than fresh WB.

(4) Leukocyte-poor red blood cells. Multiparous women (those who have had two or more pregnancies resulting in viable fetuses, whether or not the offspring were alive at birth) or patients who receive multiple transfusions may develop antibodies to leukocytes and platelets. When patients with leukocyte antibodies receive blood containing incompatible leukocytes, febrile transfusion reactions may occur. These leukocyte reactions do not cause red blood hemolysis, but can be extremely uncomfortable for the patient and are potentially fatal. Symptoms such as chills, fever, headache, malaise, nausea, vomiting, and chest or back pain may persist for up to 8 hours and seem to be caused by immune damage to donor leukocytes. The frequency and severity of leukocyte transfusion reaction is directly related to the number of incompatible leukocytes transfused. Therefore, leukocyte-poor blood (LP-RBCs) is indicated for patients who have repeated febrile transfusion reactions. Because febrile reactions occur rather commonly, patients should be switched to leukocyte-pooronly after they have experienced two or more such reactions.

(5) Frozen Red Blood Cells. The blood bank text written prior to 1978 lists decreased incidences of post-transfusion hepatitis as an advantage of frozen RBCs. Alter et al demonstrated in 1978 that human blood in which the plasma was inoculated before freezing with hepatitis B virus could transfer the virus to chimpanzees after processing, freezing, and deglycerolization. Deglycerolized RBCs have also been shown to transmit hepatitis B to human recipients. Retroviruses, such as HIV, are also likely to be present in a unit of deglycerolized RBCs. Studies are being conducted to determine if frozen blood cells prevent cytomegalovirus. Glycerolizing and deglycerolizing RBCs remove granulocytes and platelets, preventing reactions with granulocytes and platelet antibodies; however, some lymphocytes survive, thus the potential to cause graft-versus-host reactions may persist. In addition to these specific indications, the long term shelf life of frozen red cells make them ideal for storage of rare blood types and for autologous transfusion. The disadvantages of frozen RBCs are the additional cost and the limited storage period of 24 hours following thawing and deglycerolization.

c. **Effects of Red Blood Cell Transfusion.**

(1) Circulation. When a unit of WB is transfused rapidly (30 to 60 minutes) to a patient with a normal blood volume, the blood volume is increased by this amount. After approximately 24 hours, the blood volume has returned to its pretransfusion level. If plasma alone is transfused, the blood volume may readjust more rapidly. Some patients, such as those with chronic renal disease, may require prolonged periods to readjust their blood volume.

(2) Hemoglobin. The effects of red blood transfusion on the recipient's hemoglobin and hematocrit will be affected by the recipient's blood volume, pretransfusion hemoglobin and hematocrit, the clinical condition of the patient (stable, bleeding, etc), and the hemoglobin and hemacrit of the donor unit. For instance, a hypothetical patient with a blood volume of 5,000 ml and hemoglobin of 8 gm/dl has a total hemoglobin of 400 gm. If the patient is transfused with one unit of WB, the blood volume becomes 5,517 ml and the total hemoglobin 460 gm, which results in a hemoglobin concentration of 8.4 gm/dl. If red blood ceils are transfused, the blood volume is 5,300, the total hemoglobin 460 gm, and the hemoglobin concentration 8.7 gm/dl. If the patient is able to readjust the blood volume to baseline levels within 24 hours, the result will be 5,000 ml blood volume, 460 gm hemoglobin, and a hemoglobin concentration of 9.2 gm/dl with either WB or RBCs.

(3) Red blood cell production. Transfusion of red cells may result in a decrease in the recipient's own RBCl production because of a suppression of erythropoietin. Thus, many patients with a stable chronic anemia may receive little benefit from RBC transfusion since their hemoglobin rapidly fails to pretransfusion levels because of diminished production of their own RBCs.

d. **Survival of Transfused Red Blood Cells.** The normal red cell has a life span of approximately 120 days. Each unit of blood contains RBCs of all ages between 1 and 120 days. As the unit of blood is stored, the RBCs continue to age and these senescent RBCs are removed from the circulation within 24 hours, after transfusion. Thus, when stored blood is transfused, there is a slight decrease in the proportion of RBCs surviving 24 hours after transfusion. Approximately 70 percent to 80 percent of. stored in CPD for 21 days, or CPDA-1 for 35 days, survive following transfusion. The remaining RBCs survive normally, and they are destroyed linearly with a mean half-life of 50 to 60 days. The survival of transfused RBCs is affected by the recipient's health, and may be decreased in patients with active bleeding (and iatrogenic blood loss), hemolytic anemia resulting from defects extrinsic to the RBC (autoantibodies, alloantibodies, and hypersplenism), and chronic renal or liver failure.

3-3. TRANSFUSION OF PLATELETS

a. Indications for Platelet Transfusion.

(1) The decision whether to transfuse platelets depends upon the clinical condition of the patient, the cause of the thrombocytopenia, the platelet count, and the functional ability of the patient's own platelets (see Table 3-1). Patients with transient thrombocytopenia from chemotherapeutic treatment of malignancy form the largest group of patients receiving platelet transfusions. There is little risk of spontaneous hemorrhage in these patients when the platelet count is over 30,000/mm^3. Although there is some disagreement as to the value of prophylactic platelet transfusion, many physicians advise platelet transfusions to prevent serious bleeding in these patients when the platelet count is less than 20,000/mm^3.

Cause	Therapy
Amegakaryocytic thrornbocytopenia (for example, leukemia, hypopastic, or aplastic anemia)	Platelets useful in treating hemorrhage and for prophylaxis to prevent bleeding episodes
Immune thrombocytopenia purpura (for example, ITP)	Platelets of little value because of rapid destruction in the spleen.
Dilutlonal thrombocytopenia (for example, massive transfusion with bank blood)	Platelets of value in replacement (usually after 15 to 20 units transfused)
Disseminated intravascular coagulation (DIC)	Platelets of value only when combined with efforts to stop DIC or treat the cause
Functional platelet abnormalities	Platelets from normal donors may Achieve hemostasis during hemorrhage, surgery, and dental extractions

Table 3-1. Thrombocytopenia and platelet therapy.

(2) In patients with immune thrombocytopenic purpura, transfused platelets usually have a very short survival and, thus, may not be helpful. However, platelet transfusion may be effective in controlling serious active bleeding, especially in surgery. The most effective forms of treatment may be corticosteroids or splenectomy.

(3) In patients with thrombocytopenia secondary to drug idiosyncrasy, the offending drug should be discontinued and the patient closely observed. Because transfused platelets will have a shortened survival, they are advisable primarily for treatment of active hemorrhage.

b. **Outcome of Platelet Transfusion.**

(1) Some patients produce platelet antibodies as a result of previous pregnancy or transfusion. Platelets collected from random donors will have a shortened survival in those patients and, thus, may not be effective in preventing or controlling bleeding. For further discussion, see platelet compatibility below.

(2) It has been shown that from 1 to 3 hours after infusion, the platelet count increases approximately 12,000/mm^3 when 1 x 10^{11} platelets are transfused into a patient with 1 m^2 body surface area (for example., a 30-kg, 6-year-old child). A platelet concentrate usually contains approximately 0.6 x 10^{11} platelets. This would be expected to increase the platelet count approximately 4,000/mm^3 in an average adult with 1.8 m^2 surface area. Thus, if it is desired to elevate the platelet count from 5,000 to 40,000/mm^3, 9 units of platelet concentrate would be required, [(40,000--5,000) ÷ 4,000 = 9 (rounded)]. The observed increment is somewhat lower if the platelet concentrate has been stored "in vitro" before transfusion.

(3) Many patients do not show the expected increment in peripheral blood platelet count following transfusions because platelet survival is affected by the clinical condition of the patient. If active bleeding is occurring or splenomegaly exists, the transfused platelets are sequestered at the bleeding site or in the spleen and do not remain in the circulation. In patients who have platelet antibodies, such as those with idiopathic thrombocytopenic purpura or sensitization to antigens of the HLA system, survival of circulating platelets is extremely brief, sometimes only a matter of minutes. Fever, infection, and disseminated intravascular coagulation are additional clinical conditions that cause a shortened platelet survival.

c. **Selection of ABO and Rh Type for Platelet Transfusion.**

(1) ABO antigens are present on the surface of the platelet and the recovery of A_1 platelets transfused into group O patients may be decreased. In patients with lymphocytotoxic antibodies against donor cells, however, the increment in peripheral blood platelet count is the same following transfusion of HLA-compatible platelets, whether ABO-compatible or ABO-incompatible. Until these inconsistencies can be resolved, it is advisable to transfuse ABC-compatible platelets whenever possible. If ABO-compatible platelets are not available, ABO-incompatible platelets should be used rather than withholding platelet transfusions.

(2) Incompatibility between donor plasma and recipient RBCs usually is not clinically important because of the small volume of plasma (20 to 50 ml) from each individual platelet concentrate. If large numbers of platelet concentrates are being transfused to an adult or the patient is a small child, incompatible donor plasma may cause a positive direct antlglobulin test and RBC hemolysis. When group-compatible platelets are unavailable, consideration may be given to removing additional plasma from the platelet concentrate before transfusion or to giving

group-compatible plasma, rather than group-compatible platelets. Neither major nor minor crossmatch is necessary prior to platelet transfusion unless the platelet product contains many RBCs.

(3) Rh antigens are not found on platelets; however, patients may become sensitized to Rh antigens from the RBCs contaminating the platelet concentrate. The risk of forming anti-Rh_O has been shown to be approximately 8 percent, after 80 to 110 units of platelets. Because of the life-threatening nature of most cases of thrombocytopenia, platelets from Rh-positive donors can be administered to Rh-negative recipients; however, Rh-negative women in the childbearing age with a nonmalignant disease should not receive platelet concentrates from Rh-positive donors because of-the effect of possible anti-Rh on future pregnancy. Circulation of platelets from Rh-positive donors in recipients with preformed anti-Rh_O is normal; thus, the only concern is possible reaction to contaminating Rh-positive RBCs. If the platelet concentrates are properly prepared, RBC contamination is 0.4 ml or less and, thus, a red cell hemolytic reaction would not be expected even in a recipient with a preformed anti-Rh_O antibody.

d. **Platelet Compatibility.** In addition to ABO antigens, platelets contain the HLA antigens found on most tissues of the body and additional antigens that are unique to platelets. Some patients may develop antibodies to these HLA or platelet antigens following transfusion, pregnancy, or organ transplantation. When this occurs, transfused platelets have a decreased recovery and a shortened intravascular survival. These transfused platelets are ineffective in controlling hemorrhage. Compatible platelets may be obtained by HLA-matching of patient and donor. The most likely source of HLA identical or compatible donors would be the patient's family; however, large files of HLA-typed donors are being developed for clinical research purposes. HLA-matching of patients with unrelated donors may become practical. HLA-matching using the lymphocytotoxicity assay may not be the best method of determining platelet compatibility; however, it is the only one currently available on even a limited scale. Thus, in patients who are unresponsive to the transfusion of platelets collected from random donors, selection of donors based on HLA-typing may provide platelets with better posttransfusion recoveries and survival.

e. **Administration of Platelets.**

(1) Patients with platelet or HLA antibodies may have febrile nonhemolytlc reactions to Incompatible platelets. These reactions may be caused by incompatible platelets or by leukocytes that invariably contaminate the platelet preparation. In addition, platelets may be trapped in the pulmonary capillaries, causing dyspnea and pulmonary edema. This is particularly likely if aggregates of platelets are infused. If the platelets are properly prepared, they will contain very few aggregates.

(2) Platelet administration sets contain a filter that is similar to a standard blood filter, but housed in a small drip chamber, or a needle syringe device. Only approximately 3 percent of platelets are lost by passage through these filters. Microaggregate filters may trap a large proportion of platelets, and should not be used for platelet administration.

3-4. BLOOD PRODUCTS USED TO REPLACE PLASMA COAGULATION FACTORS

a. **Single Coagulation Factor Deficiency.**

(1) Factor VIII.

(a) Deficiencies of coagulation factors may exist as isolated or combined deficiencies and may be acquired or inherited. Isolated inherited deficiencies of each at the coagulation factors have been described, although the most common is hemophilia A, or Factor VIII deficiency. Opinions differ regarding the level of Factor VIII that is desirable to be attained in the management or prevention of bleeding episodes.

(b) One unit of Factor VIII equals the Factor VIII activity of 1 ml of fresh, normal, pooled plasma. Factor VIII levels are usually reported as a percentage of normal (see Table 3-2).

Blood Components	Volume ml	Units* Factor VIII per Container	Units* Factor VIII per ml
Fresh whole blood (24 hr)	517.5	225	1.0
Fresh liquid plasma	225	225	1.0
Fresh-frozen plasma	225	190	0.8
Cryoprecipitate	10	100	10.0
Commercial concentrate	20 to 30	200 to 1,000	10 to 33

(*One unit of Factor VIII is that amount of Factor VIII activity in 1 ml of fresh normal plasma.)

Table 3-2. Blood components and fractions containing factor VIII.

(c) The amount of Factor VIII required for transfusion can be calculated as follows:

$$\frac{1}{} \text{ Weight (kg) x 70-ml/kg blood volume (ml)}$$

2 Blood volume (ml) x (1 -hematocrit) = plasma volume (ml)

3 Plasma volume (ml) x (desired Factor VIII level u/ml - initial Factor VIII u/mi) = units Factor VIII required

Example: To raise the Factor Viii level to 50 percent in a 70-kg patient with a hematocrit of 40 percent and a factor VIII level of 0 percent.

70-kg x 70 ml/kg = 4,900 ml

4,900 ml x (1-0.40) = 2,940

2,940 ml x (0.5-0) = 1,470 units

(d) In cases where the initial Factor VIII level is not known, it can be assumed to be 0 percent in a patient with severe classic hemophilia A. The half-life of Factor VIII after transfusion is 8 to 12 hours, so that it is usually necessary to repeat the Factor VIII transfusion at 8- to 12-hour intervals in order to maintain hemostatic levels. Some hemophiliacs may have an inhibitor that causes a shortened half-life of Factor VIII after transfusion. In calculating the dose of Factor VIII; it may be necessary to account for other variables, such as differences in the intravascular half-life of transfused Factor VIII and the extravascular distribution of Factor VIII. The calculations, described above, provide an estimate of the Factor VIII level attained immediately after transfusion. Dosage should be adjusted so that the minimum desired level is reached just prior to the next infusion.

Example: If the desired minimum Factor VIII level = 30 percent and since the half-life of Factor VIII = 12 hours, it is necessary to elevate the patient's initial Factor VIII to 60 percent so that the level just before the next dose will be 30 percent.

Patient's plasma volume = 2,940 ml

2,940 ml x (60-0) or 1,764 units required to elevate Factor VIII to 60 percent

12 hours later, half of the Factor VIII remains, thus: the Factor VIII level is 30 percent

For the next dose of Factor VIII, 2,940 x (0.60-0.30) or only 882 units are required to elevate Factor VIII to 60 percent

(e) Once the dose of Factor VIII has been determined, the amount of product necessary can be calculated easily (see Table 3-2). The duration of treatment with Factor VIII depends upon the type and location of the hemorrhage and the clinical response of the patient.

(f) In addition to cryoprecipitated Factor VIII and fresh-frozen plasma, a number of lyphilized preparations are available for treatment of Factor VIII deficiency. These preparations are assayed and labeled for Factor VIII activity, and can be stored at 4ºC for extended periods. Since they are made from large plasma pools, they may carry a higher risk of hepatitis than a comparable amount of Factor VIII administered as cryoprecipitate.

(2) Factor IX.

(a) Isolated inherited deficiency of Factor IX is called hemophilia B, and is clinically similar to hemophilia A. Factor IX Is stable when stored at 4ºC, or at -20ºC. Thus, bank blood, liquid plasma, or fresh-frozen plasma can be used to replace Factor IX; however, it is difficult to replace large amounts of Factor IX because of the volume of these products.

(b) Within the past few years, commercial preparations containing concentrated Factor IX (II, VII, IX, X complex) have become available and can be used when large amounts of Factor IX must be administered. Disease transmission is a major risk of transfusion of Factor IX concentrates.

(3) Fibrinogen.

(a) Hypofibrinogenemia may occur as an isolated inherited deficiency, or it may be acquired associated with obstetrical complications, disseminated intravascular coagulation, and some forms of cancer. In acquired hypoflbrinogenemia, treatment should be directed toward the underlying cause of disease rather than toward replacement of fibrinogen. Many physicians provide fibrinogen replacement during correction of the underlying disorder.

(b) Commercial fibrinogen preparations, formerly available, are no longer manufactured because at the high risk of transmitting hepatitis B. Cryoprecipitate is used as a source of fibrinogen for replacement therapy. Each bag of cryoprecipitate from a single donor contains approximately 250 mg of fibrinogen. A quality assurance program should be established so that this fibrinogen content will be known in each unit of cryoprecipitate.

b. **Deficiency of Multiple Coagulation Factors.**

(1) The most common combination deficiency of coagulation factors involves those dependent upon vitamin K for synthesis. Deficiency of these factors (prothrombin, VII, IX, X) most commonly occurs in patients with liver disease or lack of vitamin K. Inhibition of vitamin K may occur when excessive amounts of oral anticoagulant drugs (coumarin) have been taken. Vitamin K deficiency may occur when intestinal fiora are reduced (neonates, antibiotic therapy) in malabsorption syndromes or when bile fails to reach the intestinal lumen (bile duct obstruction, biliary fistula). This type of coagulation disorder is best managed by treating the underlying condition with or without vitamin K administration; however, the coagulation factors can be replaced using plasma of any age since those coagulation factors do not deteriorate during storage of WB at 1ºC to 6ºC.

(2) Commercial concentrates containing Factor IX (II, VII, IX, X complex) should not be used to replace an acquired deficiency of multiple factors because of the high risk of hepatitis that is associated with these concentrates.

c. **Administration and Blood Group Compatibility of Products Used to Replace Coagulation Factors.**

(1) In the transfusion of plasma products, procedures of patient blood product identification, venipuncture, infusion solutions, and the use of filters are the same as described for RBC transfusion. Since serious reactions may occur during the transfusion of plasma products, the nurse should obtain the patient's vital signs before initiating transfusion. The patient should be reevaluated approximately 15 minutes later to ensure that the transfusion is proceeding uneventfully, and should be evaluated at the end of transfusion to determine whether any adverse reaction has occurred. The rate of administration should be as rapid as possible, but this depends upon the patient's ability to tolerate the volume being infused. Fresh-frozen plasma need not be ABO-identical, but should be compatible with the recipient's RBCs and can be given without regard to Rh type. Compatibility testing is not necessary if fresh frozen plasma has been tested for unexpected RBC antibodies.

(2) Cryoprecipitate should also be administered as ABO-compatible whenever possible. Although the volume of each unit is small, most therapy involves many units and, thus, the volume of plasma being infused becomes significant. Cryoprecipitate can be administered without regard to Rh type. While compatibility testing is not necessary ABO-incompatible cryoprecipitate and commercial concentrated preparations of Factor VIII contain anti-A and anti-B, which may cause a positive direct antiglobulin test and/or a hemolytic anemia if massive doses are administered. In addition, the recipient's fibrinogen may become elevated by the fibrinogen contained in the preparation.

3-5. PLASMA SUBSTITUTES

a. In recent years, plasma substitutes have become popular since they provide volume and colloid without the risk of hepatitis or acquired immunodeficiency syndrome (AIDS). Five percent normal serum albumin in saline and five percent plasma protein fraction (PPF) are available in 250-ml and 500-ml containers (see Table 3-3). Except for electrolyte concentrations, they are quite similar. Both are useful for the treatment of hypovolemic shock, burns, and other clinical conditions in which both volume and colloid must be replaced. Albumin is also available as a concentrate with little sodium chloride added (25 percent salt-poor albumin). Considerable caution must be used since the albumin concentration may raise the oncotic pressure in the vessels dramatically, drawing large volumes of water from the tissues into the vascular space. This may, in turn, produce cardiac overload.

b. The value of any of these albumin solutions in the treatment of chronic hypoalbuminemic states, such as cirrhosis, protein losing enteropathy, or cachexia, has not been established.

	Vol. (ml)	Albumin	Globulin	Na	K	Cl	Stable	Labile	Hepatitis Risk	Sensation Risk	X-match Interference	Storage (yr)
Plasma preparations												
Single donor	250	8.0-13.0	2.0	35	1	25	+		Yes	Occasionally	No	5
Plasma		8.0-13.0	8.0									
Single donor, fresh	250	8.0	2.0	35	1	25	+	+	Yes	Occasionally	No	1
Frozen plasma		13.0	8.0									
Heat-treated protein preparations												
Normal serum albumin (salt-poor)	250	12.5	-	35	0	25	-	-	No	Rare	No	3
Plasma protein	250	11	1.5	22.5	0.5	12.5	May cause bleeding		No	Rare	No	3
Fraction												
Plasma substitutes												
5 percent dextral 70	250	-	-	35	1	35	May cause bleeding		No	Yes	Possible	4

Table 3-3. Plasma substitutes. (Continued)

Vol. (ml)	Albumin	Globulin	Na	K	Cl	Stable	Labile	Hepatitis Risk	Sensation Risk	X-match Interference	Storage (yr)
Gelatin 250	-	-	35	- 35	May cause bleeding		No	Yes	Possible	1	
Hyroxyethyl starch 6 percent 250	-	-	35		May cause bleeding		No	No	Possible	7	
Crystalloid 250	-	-	35	1 27	-		No	No	No	Indef	
Lactated Ringer's											

Table 3-3. Plasma substitutes. (Concluded)

3-6. SPECIAL SITUATIONS INVOLVING TRANSFUSION

a. **Massive Transfusion.**

(1) Massive transfusion can be defined as transfusion of the patient's blood volume during a 12-hour interval. The effects of massive transfusion upon the recipient may be due to the biochemical and functional characteristics of stored blood:

(a) Platelets deteriorate during storage of WB.

(b) Coagulation Factors V and VIII deteriorate during storage of WB.

(c) The oxygen saturation curve of hemoglobin shifts and oxygen is less readily released to the tissues.

(2) Transfusion of large amounts of blood depleted of platelets and Factors V and VIII may create deficiencies in the recipient because of dilution of the recipient's blood with this depleted stored blood. However, usually Factor VIII is rapidly replaced by the patient and Factor V levels do not fall below that needed for hemostasis. In addition, the hemostatic process that occurs in the bleeding patient consumes the patient's own platelets and coagulation factors and compounds the depletion state. Oxygen delivery by transfused cells stored more than two weeks in CPD may be diminished immediately following transfusion, but oxygen release improves approximately 24 hours following transfusion.

(3) Patients undergoing massive transfusions should be followed closely with coagulation studies, including a platelet count. If coagulation abnormalities or thrombocytopenia develop, these deficiencies should be replaced with the appropriate blood components. It is usually not possible to correct these deficits with fresh WB. Fresh-frozen plasma, platelet concentrates, and RBCs will also be more readily available than fresh WB. Coagulation Factor IX concentrates should not be used in these situations (see above).

(4) Despite dilution of the patient's blood with donor plasma, continued compatibility-testing is recommended. If it is necessary to change to a different blood group in massive transfusion, the patient's history and clinical situation should be considered, as well as the potential blood supply. It is sometimes more desirable to switch Rh types (for instance from Rh-negative to Rh-positive) than to switch ABO group. However, age and sex may also be important to consider. For example, when transfusing a young, Rh-negative woman, it is usually preferable to switch ABO groups, if feasible, before switching Rh.

(5) The likelihood of development of hypocalcemia resulting from the infusion of large amounts of citrate during massive transfusion has been overemphasized. Administration of calcium during massive transfusion is probably not necessary.

(6) Warming of blood may be necessary if large amounts of blood are being transfused rapidly. Procedures for warming blood are discussed below.

b. **Pediatric Transfusions.**

(1) Children who are not actively bleeding should receive RBCs for the same reasons that RBCs are superior to WB for adults. If transfusions of small volumes of blood are to be administered, 1 donor unit can be collected into a multiple container and divided into small volumes as needed.

(2) Premature and newborn infants usually require very small volumes of blood, such as 30 to 60 ml of WB or RBCs, although an additional 30 ml is required to fill the administration set. In some hospitals, this has led to the use of "syringe" transfusions from "walking" donors. Syringe transfusions are not desirable because (1) the donor medical history is usually inadequate, (2) pretransfusion testing of donor blood is usually not performed, (3) compatibility testing is usually not performed, (4) proper ratio of anticoagulant is often not used, (5) no filters are used for administration, (6) identification systems are often inadequate, and (7) risk of CMV hepatitis or AIDS from untested blood.

(3) Transfusion of small volumes of blood should be carried out through the facilities of the blood bank where well-trained personnel perform proper medical histories and pre-transfusion and compatibility testing of the blood. Blood for these special transfusions can be collected into a multiple bag and divided; or 480 to 490 ml can be collected into a double bag and 30 to 60 ml removed into the satellite bag for pediatric transfusion. Small collection containers with CPD anticoagulant in 150 ml primary with a 150 ml satellite bag are now available. Thus, one donor could give up to three times during a 2-month interval, and each donation split into 2 to 4 parts.

(4) Compatibility testing for neonates (newborn children less than a year old) is different from those for adults. Initial testing must include ABO and Rh typing of the neonatal recipient's RBCs. An antibody screening test, which may be done either on the newborn or mother's serum or plasma, is also done. If the antibody screen is negative and group O or ABO specific, or compatible with both mother and child, to include the same Rh type, then compatibility testing and further typing may be omitted during the first 4 months of life.

c. **Granulocyte Transfusions.** Special instruments have been developed that make granulocyte collection possible. Transfused granulocytes function normally and, in selected patients, produce clinical benefits. Patients with profound neotropenia or chronic granulomatous diseases are likely to receive granulocyte transfusions. Granulocyte transfusions have not been proven effective in patients with localized infections or infections with agents other than bacteria. Those recipients who survive this acute infection can be expected to have a satisfactory quality of life for a reasonably long period of time.

d. **Opsonins in Stored Blood.** Opsonic activity is stable in CPD blood stored under standard conditions for 28 days. Fibronectin is also stable in stored blood products. Patients receiving massive transfusions with RBCs, crystalloids, or albumin solutions may have diminished circulation opsonins. Some personnel have suggested that raising plasma opsonin levels might be beneficial. However, specific therapeutic recorrmendations must await controlled clinical studies and further understanding of the roles of these proteins in health and disease.

3-7. ADMINISTRATION OF BLOOD PRODUCTS

NOTE: Once a blood transfusion has been ordered, the procedure should be explained to the patient in order to minimize his apprehension. The following steps are important to ensure a safe and efficient transfusion.

a. **Obtaining the Sample To Be Used For Compatibility Testing.** See Lesson 1, Section IV, Determination of Compatibility.

b. **Blood Administration Sets.**

(1) Red blood cells, platelets, granulocytes, fresh-frozen plasma, and cryoprecipitate should be administered through a filter because fibrin clots and other particulate debris may be present. Most standard blood and platelet filters have a pore size of approximately 170 to 260 micrometers, but there is some variation in the surface area of the filter and the arrangement of the filter and drip chamber. Filters with a larger surface area may allow more rapid infusion of RBCs because, although the pore size is the same, there is more filtration area. The filter chamber should be filled with blood in order to utilize all this surface area. The frequency with which filters should be changed depends upon the type of blood product being infused and, if RBCs are involved, the age of the product. As debris accumulates on the filter, the rate of infusion is slowed. In addition, platelets may adhere to the debris on the filter.

(2) A single filter can usually be used for administration of 2 to 4 units of RBCs. Because of the hazards of hemolysis and bacterial contamination, once a filter has been used and contains blood or debris, it should not be left for extended periods and then reused.

c. **Microaggregate Filters and Microemboli.**

(1) Recently, there has been considerable interest in the particles that develop in stored blood and are too small to be removed by standard blood filters. The presence of these microaggregates can be measured by the screen filtration pressure (SFP) which is the pressure required to force blood through a mesh filter with 20-micrometer pores. Patients who receive massive transfusions of older stored blood have a progressive fail in SFP from central venous to arterial blood, suggesting that the transfused microaggregates lodge in the pulmonary microcirculation. This has been suggested as an etiologic mechanism of shock lung, with its attendant hypoxemia and pulmonary microemboli; however, patients receiving massive transfusions usually have many complicating factors and the exact role of these microaggregates in humans remains unsettled.

(2) There are five different types of filters: (1) screen filtration, (2) dacron wool filtration, (3) polyurethane foam filtration, (4) polyurethane foam plus nylon wool filtration, and (5) screen filtration plus Dacron wool. Commercially available microaggregate filters are composed of one or more of these materials. The relative ability of these filters to trap microaggregates has not been established. As the filter becomes saturated with microaggregates, the flow rate of blood through it decreases. Thus, if a microaggregate filter is to be used, the selection of a specific brand is often a matter of balancing the degree of microaggregate removal against the flow rate desired through the filter and the frequency with which the filter can be changed. Most, if not all, of these filters remove platelets and, therefore, are contraindicated for use in the transfusion of fresh WB, platelet concentrates, or granulocyte concentrates. Other hematologic parameters remain unchanged after passage of blood through microaggregate filters; however, it seems reasonable that infusion of too many units through the same filter, especially if pressure is used, may cause RBC hemolysis.

(3) Microaggregate filters are not required for most transfusions. If the blood bank physician feels that microaggregate filters are indicated, a general policy can be established, such as instituting their use in a particular patient after a certain number of transfusions within 12 or 24 hours. Microaggregate filters are routinely used during cardiopulmonary bypass.

d. **Venlpuncture.**

(1) Blood products should be administered intravenously, although other routes (intra-peritoneal, intra-arterial, intrabone marrow) are possible. A vein should be selected which will be large enough to accommodate the infusion needle, but is comfortable for the patient. Veins In the anticubltal fossa are probably more accessible and most widely used; however, infusion in these veins limits the patient's ability to flex the elbow during transfusion. Veins on the forearm or hand are equally suitable for infusion, although venipuncture in these areas is often more painful to the patient.

(2) The administration set should be cleared of air before venipuncture. Venipuncture can be performed with a needle attached to a syringe or attached directly to the blood administration set. Red blood cells or WB should be administered using a needle of 19 gauge or larger. Other blood products such as platelets, cryoprecipitate, fresh-frozen plasma, and albumin can be administered through smaller needles.

(3) For pediatric patients, blood is often infused through a 23-gauge, thin wall, scalp vein needle. Because the RBCs may run slowly through a small needle, the unit may be divided in the blood bank. One part can be released for transfusion and the remainder of the unit stored in the blood bank. This prevents prolonged exposure of the RBCs to room temperature.

e. **Issuing the Blood Product.**

(1) The venipuncture should be started before, or at the time, the blood component is being obtained from the blood bank. Thus, the blood component can be infused immediately after it has arrived at the nursing station, minimizing the chance of improper storage after the component leaves the blood bank.

(2) When the blood component is released from the blood bank the technologist should:

(a) Compare the ABO, Rh type, and unit number on the component labels with the same information on the compatibility or recipient tag.

(b) Compare the product name with the blood request form to be certain the component being released is the same as that ordered by the physician.

(c) Record the name of the Individual to whom the component was released.

(d) Carry out the steps as in paragraph 3-7a and b above.

f. **Starting the Transfusion and Infusion Solutions.**

(1) Blood products, except platelets and thawed cryoprecipitate or fresh-frozen plasma, should be stored in a regulated blood bank refrigerator until immediately before transfusion. Do not place blood components in the ward refrigerator or near a cold window since freezing and thawing will cause RBC hemolysis. If the transfusion cannot be started shortly after the blood arrives at the nursing station, it should be returned to the blood bank for storage. Since it is impossible to monitor the temperature of the blood while it is outside the blood bank and to be sure that the blood has not reached a temperature higher than 10ºC, it is customary to establish a time limit

within which blood may be out of the control of the blood bank and returned for reissue. One study showed that blood in a room temperature environment required approximately 30 minutes to warm from 2ºC to above 6ºC.

(2) Sodium chloride injection USP (normal saline) is the only solution suitable for use in the transfusion of blood products containing RBCs, platelets, or leukocytes. “In vivo” hemolysis of RBCs exposed to various IV solutions seems to be primarily dependent upon the amount of RBC swelling that occurs in vitro”. Five percent dextrose in water is not satisfactory for filling or flushing blood administration sets because RBC clumping and swelling with subsequent hemolysis may occur. Lactated Ringer’s solution also is unsatisfactory because calcium in the Ringer’s solution may cause the formation of clots. Great care must be taken to ensure that drugs which are toxic to blood components are not infused through the same administration set as the blood component.

(3) Sometimes it is difficult to infuse RBCs because the high hematocrit decreases the flow rate. This problem can be avoided by adding normal saline to the RBC units at the time the transfusion is started. The technique is as follows:

(a) A Y-type infusion set must be used.

(b) Perform the venipuncture and begin the infusion with normal saline connected to one lead of the Y infusion set.

(c) The second lead of the Y infusion set should be clamped closed.

(d) Connect the unit of RBCs to the second lead of the Y set.

(e) Lower the unit of RBCs below the bottle of normal saline.

(f) Open the clamp on the lead to the RBCs and allow the desired volume of saline to enter the unit of RBCs. This volume will usually be 50 to 100 ml.

(g) Clamp the lead coming from the saline bottle.

(h) Hang the unit of RBCs diluted with saline beside the bottle of normal saline.

(i) If it is desirable, the RBCs remaining in the infusion set at the end of transfusion can be rinsed into the patient by opening the lead from the saline bottle.

g. **Identification of Patient and Blood Product.**

NOTE: Before beginning the transfusion, it is extremely important to identify correctly the patient and the blood product. It is ideal for two persons to carry out the steps listed below, thus cross-checking the information.

(1) Identification of the blood product.

(a) Check the ABO group and Rh type on the label on the blood container to be certain it agrees with the compatibility record.

(b) Check the number on the label on the blood container to be certain it agrees with the compatibility record.

(c) Check the blood compatibility record for the patient's name and hospital number.

(2) Identification of the patient.

(a) Check the name and hospital number on the patient's wrist identification band against the information on the compatibility record.

(b) When possible, ask the patient to identify himself by stating his name. Never ask, "Are you Mr. _____?"

(c) The person who identifies that the correct blood product is being administered to the patient should then sign the compatibility record, and that record should be placed in the patient's chart at the completion of the transfusion.

NOTE: Do not begin the transfusion until any discrepancy in the above information is resolved.

h. **Rate of Infusion.**

(1) The rate of infusion depends upon the clinical condition of the patient and the product being transfused. In most administration sets, 15 drops equals 1 ml. Most patients who are not in congestive heart failure or in danger of fluid overload tolerate the infusion of one unit of RBCs in 1 1/2 to 2 hours.

(2) The transfusion should be completed in less than 4 hours because of the dangers of bacterial proliferation and RBCs hemolysis at room temperature. If the desired volume of RBCs will not be infused within 4 hours, the original unit should be divided and one portion stored in the blood bank until it is needed.

i. **Nursing Care.**

(1) Proper nursing care is important for patients receiving transfusions of all blood components, including platelets, plasma products, cryoprecipitate, and albumin, as well as RBCs.

(2) Base line values for temperature, pulse, respirations, and blood pressure should be obtained before beginning transfusion. During the first 15 minutes, the rate of infusion of RBCs should be very slow, approximately 100 ml per hour. This will minimize the volume of RBCs infused if the patient experiences an immediate reaction. The nurse should observe the patient during at least the first 5 minutes of transfusion, and then return after 15 minutes to ensure that the transfusion is proceeding uneventfully. If so the rate of infusion can then be increased, to that ordered by the physician.

(3) At the termination of the transfusion, the nurse should record whether an adverse reaction has occurred and discontinue the intravenous infusion. All adverse reactions should be reported to the blood bank as soon as practically possible. If other intravenous therapy is ordered after the blood transfusion, the infusion set should be rinsed with normal saline before starting these drugs or solutions.

j. **Warming of Blood.**

(1) It is not necessary to warm blood before transfusion except in unusual circumstances, such as (1) massive transfusions or when the infusion rate is greater than 50 ml/per minute, (2) occasionally in exchange transfusion of the newborn, or (3) patients with potent cold agglutinins.

(2) If blood must be warmed prior to transfusion, this should be performed by passing the blood through coils immersed in a water bath or dry incubator where the temperature is maintained at approximately 35ºC, but always less than 38ºC. Hemolysis may occur when blood is subjected to temperatures greater than 40ºC. Blood should never be warmed by placing it near a radiator, heater, or stove. Microwave instruments are available for warming blood, but these instruments have malfunctioned, causing hemolysis of the RBCs being warmed. If any warming device is utilized, it should undergo careful and continuing quality assurance procedures and the staff should be thoroughly trained in its operation. A blood-warming device that is not of the in-line type should be located in or under the control of the blood bank. Once blood has been warmed, it cannot be returned to the blood bank for future transfusion. It is desirable to indicate on the transfusion that the blood component has been warmed.

Section II. ADVERSE EFFECTS OF BLOOD TRANSFUSION

3-8. BACKGROUND

a. A transfusion reaction is any unfavorable event occurring in a patient during or following transfusion of blood products which can be related to that transfusion. Since compatibility testing is performed for the detection of antibodies to RBC antigens, adverse effects of transfusion are most commonly caused by leukocytes, platelets, and plasma proteins. In addition, every transfusion carries a risk of alloimmunization as well as transmission of disease. All the care in cross-matching blood in the laboratory can be negated by the administration of blood to the wrong patient.

b. All transfusion reactions should be reported to the blood bank and evaluated to the extent considered appropriate by its Medical Director. Major adverse effects, for example, hemolytic transfusion reactions and disease transmission, must be reported to the Bureau of Biologics, Food, and Drug Administration.

c. Whenever a transfusion reaction involving more than just hives is suspected, the transfusion should be immediately discontinued, but the intravenous line kept open. The remaining blood, a new sample from the recipient, plus the reaction report, should be sent to the blood bank for prompt investigation. The detailed clinical management of adverse effects of blood transfusion may be obtained elsewhere, but Table 3-4 contains suggested treatment regimens.

FINDINGS	TREATMENT
Urticaria (hives) only Fever and/or chills	Intramuscular antihistamines
1. Examine patient's blood for: Intravascular hemolysis (plasma red or pink caused by free hemoglobin) or extravascular hemolysis (direct globulin test). 2. Examine donor plasma for bacteria and submit for culture	1. Stop transfusion, keep I.V. open. 2. If laboratory tests are negative, treat with antipyretics. With positive findings, start prophylactic treatment as below.
Shock, hemoglobinria, oliguria, and/or diffuse bleeding.	1. Stop transfusion, keep I.V. open. 2. Maintain blood pressure with vassopressor, if necessary. 3. Maintain urine flow over 100 ml/hr a. Mannitol 25 gm I.V. diuretic b. Fluids 4. Replace clotting factor deficits when indicated, for example, with fresh-frozen plasma and/or platelets as appropriate. 5. Antibiotics and hydrocortisone for septic shock.

* Infuse IV 100 cc of 25 percent mannitol solution within a 15-minute period. This dose will initiate a diuresis, of I to 3 ml of urine per minute, in an adequately hydrated patient. The same dose may be repeated, if urine flow drops below 100 ml per hour, for any subsequent 2-hour period. Mannitol may be discontinued, when the patient can maintain a urine flow of 100 ml per hour without its use.

SPECIAL NOTE: If the history, physical findings, and clinical course are such that a hemolytic transfusion reaction is suspected as highly probable, mannitol infusion should be started, even prior to or concurrent with laboratory investigation, since under the conditions of use prescribed above, no direct adverse sequelae from the use of mannitol will occur. If diffuse bleeding is because of disseminated intravascular coagulation, consider using heparin before replacing clotting factor deficits.

Table 3-4. Management of transfusion reactions.

3-9. IMMEDIATE EFFECTS

a. **Circulatory Overload.** Sudden increases in circulating blood volume are not well tolerated by certain patients, for example, infants and individuals with chronic anemia. Whole-blood transfusion or volume expanders, like 25 percent albumin, may precipitate congestive heart failure manifested by coughing, cyanosis, and difficulty in breathing. Congestive heart failure because of circulatory overload may be the most preventable adverse effect of transfusion therapy, although it is infrequently reported to the laboratory. Patients susceptible to circulatory overload should be transfused with concentrated RBCs at a rate no faster than one ml per kilogram of body weight per hour.

b. **Febrile Nonhemoiytic Reactions.**

(1) Febrile reactions, often preceded by chills, constitute the bulk of transfusion reactions investigated by the blood bank. These reactions are generally considered to be a result of cytotoxlc or agglutinating antibodies in either donor or recipient plasma directed against antigens present on lymphocyte, granulocyte, or platelet cell membranes. While reactions are usually mild and result principally in recipient anxiety and discomfort, in rare instances pulmonary infiltrates, leukopenia and shock, or even death has been reported. Leukocyte-poor (or frozen, thawed, washed) RBCs blood cells should probably be given to recipients who display repeated chill/fever reactions to transfused RBCs.

(2) Chills and fever, primarily a result of the leukocytes contaminating platelet concentrate preparations, may be seen in patients who receive repeated platelet transfusions. Removal of leukocytes from platelet concentrates may diminish febrile responses in immunized recipients and improve post transfusion platelet recovery and survival. The use of HLA-compatible or identical donors may also be effective in preventing reactions and in improving post-transfusion platelet recovery and survival in immunized recipients. Chill/fever reactions are frequently seen during transfusion of granulocytes collected by filtration leukapheresis, and to a lesser extent, those prepared by differential centrifugation.

c. **Allergic Reactions.**

(1) Allergic reactions following blood or plasma transfusions occur less frequently than leukocyte chill/fever reactions and are usually relatively mild. Most consist of local erythema, hives, and itching which develop during transfusion and that can be easily treated with, or prevented by, administration of antihistamines.

(2) More severe reactions characterized by flushing, nausea and vomiting, diarrhea, changes in blood pressure, and frank anaphylaxis have been reported in persons without immunoglobulin A (lgA). These patients have developed lgG antibodies against lgA and react to all blood products containing lgA, for example, plasma. Patients with known anti-lgA antibody should be transfused only with blood or plasma obtained from themselves or from other lgA-deficient donors or with extensively washed RBCs.

NOTE: The American National Red Cross, Washington, D.C., AABB Rare Donor file, and Canadian Red Cross, Toronto, Ontario, maintain registries of lgA-deficient donors. While an antihistamine, for example, diphenhydramine (Benadryl), may be sufficient for some allergic reactions, use epinephrine for any anaphylactic reactions.

d. **Hemolytic Transfusion Reactions.**

(1) Hemolysis of transfused RBCs occurs infrequently, but may cause a severe reaction accompanied by hemoglobinemia, hemoglobinuria, hypotension, disseminated intravascular coagulation, acute renal failure, and death. Initial recipient symptoms are not diagnostic of hemolysis and often consist of flushing, a feeling of apprehension, chest or back pain, chills, fever, and nausea or vomiting. During anesthesia, the development of diffuse bleeding may be the only evidence of a hemolytic reaction. Red blood cell destruction may be primarily intravascular, as seen with ABO-incompatible RBC infusion or predominantly extravascular as in Rh incompatibility. Intravascular hemolysis usually occurs much more rapidly, and is more likely to result in recipient harm than the relatively slow extravascular removal of RBCs by the reticuloendothelial system.

(2) All transfusion reactions should be investigated, primarily to detect the small number of reactions in which there is hemolysis (primarily caused by destruction of transfused erythrocytes). The investigation, described below, is applicable to most transfusion reaction workups (see Table 3-5). If a reaction occurs that involves more than just urticaria, the blood infusion should be stopped immediately but the intravenous line should be kept open, for example, with physiologic saline. If urticaria (hives) is the only manifestation of a transfusion reaction, treatment with an antihistamine will usually suffice; this is the only situation in which the blood can continue to be infused. Next, a properly identified sample of blood (preferably an anticoagulated one and a clotted one) obtained from the recipient, the blood bag (clamped or sealed off), and the compatibility slip should be sent to the blood bank with a description of the transfusion reaction.

(a) The first thing blood bank personnel must do is a "clerical" check of the labels and preissue records. If the "wrong" unit of blood was issued, much of the further testing and activity may be totally unnecessary. Record these "paper" findings. Representative forms for the clinical and laboratory evaluation of suspected reactions are at the end of this lesson.

1. Clerical check for paper" errors, for example, blood to the wrong patient.

2. Specimens needed:

   a. Pre-reaction blood of recipient.
   b. Post-reaction blood of recipient (anticoagulated and clot tubes).
   c. Blood from integral donor tubing or container implicated in reaction.
   d. Post-transfusion urine (spun specimen).

3. Investigation procedures (letters refer to specimens listed above):

Immediate	Definitive	Corrobative
Examine for visible + hemolysis (a, b, c, d).	Repeat crossmatch {a, b, c, (major and minor)}.	Identification of any unexpected antibody or incompatibility. Bacteriologic smear and culture (c).
Repeat ABO (a, b, c). Repeat Rh (a, b, c). Direct antiglobulin test (a, b).	Repeat antibody-screening (a, b, c).	Optional: haptoglobulin (a, b). methemalbumin (a, b). bilirubin (b). creatinine Direct antiglobulin test (c).

Adapted from Huestis, Bove, and Busch. With permission from Little, Brown, and Co.

* The procedures and specimens listed are applicable to most situations. Of course, circumstances may vary and require different approaches in particular cases.

* Examine the anticoagulated tube from b as it is less likely than the clot tube to show spurious "in vitro" hemolysis and can be more rapidly evaluated (spin and observe).

Verify the presence of the implicated rbc antigen on the donor cells (c) and the lack of this antigen on the recipient's cells (a) for any identified antibody in (b). Absence of a donor antigen post-transfusion, which the patient lacked pretransfusion, is also evidence that the donor unit has been eliminated; this may also be reflected In the failure of the anticipated rise in hemoglobin in the recipient after the transfusion.

Table 3-5. Schedule of investigation of suspected transfusion reactions.

(b) Second, the post-reaction, anticoagulated sample should be examined for evidence of hemolysis and the direct antiglobulin test performed. If there is no hemolysis and the direct antiglobulin test is negative, it is unlikely that a hemolytic reaction has occurred; however, if hemolysis is noted in the post-reaction specimen or if the direct antiglobulin test is positive (and was negative prior to transfusion), the physician caring for the recipient should be notified immediately so that he may begin appropriate therapy. Then the blood bank should search further for the etiology.

(c) Third, a complete evaluation should be initiated. This evaluation should include major (and, if necessary, minor) cross-matches using both pre-reaction and post-reaction serum samples versus RBCs from integral tubing or obtained from inside the blood bag. The ABO (direct and reverse) and Rh type of the recipient and the cells in the bag should also be (re)determined; the plasma in the bag should be examined for the presence of hemolysis that may indicate improper handling of the blood following collection or the presence of bacterial contamination. If the latter is suspected, the unit of blood should be cultured at both 37ºC and at room temperature. Remember, hemolysis may occur without serologic evidence of incompatibility.

(3) Two minor points to keep in mind regarding evaluation of a hemolytic transfusion reaction are: (1) testing for haptoglobin and (2) follow-up serologic tests if no RBC antibody is detectable. With visible hemolysis, the haptoglobin-binding capacity of serum is already exceeded and the level is nil. Documenting this absence of haptoglobin is thus rarely of value. If no antibody is detected at the time of hemolysis because of immune destruction of RBCs, it may have all been consumed during the reaction. Testing serum samples drawn a few days later will often reveal the amnestic return of the antibody.

e. **Reactions Caused by Bacterial Contamination.**

(1) Contamination of blood or components with bacteria occurs very rarely. Transfusion of blood with bacteria may produce a severe and life-threatening reaction, characterized by the rapid onset of chills, high fever, vomiting, diarrhea, marked hypotension, and often-acute renal tubular necrosis. In the past, most severe reactions were caused by gram-negative organisms capable of proliferating at refrigerator storage temperatures; gram-positive organisms were infrequently implicated, presumably because of their inability to multiply at those temperatures. Open procedures for leukapheresis, plateletpheresis, RBC glycerolization, and deglycerolization all have the potential for introduction and proliferation of bacteria; components prepared by these techniques must be used within 24 hours. Contamination of I.V. solutions or wash solutions used with blood components should also be considered when a reaction to bacteria is suspected.

(2) Blood should be examined routinely to detect evidence of bacterial contamination; look for an unusual color or the presence of hemolysis. If bacteria are suspected to be the cause of a transfusion reaction, the blood from the bag, the patient, and all IV solutions used should be cultured. Although the appearance of microorganisms on a gram-stained smear provides proof of bacterial presence, the absence of visible microorganisms does not rule out the possibility of contamination of a blood product.

f. **Other Untoward Effects of Transfusion.**

NOTE: The rapid administration of a large volume of blood may be associated with undesirable consequences.

(1) Hypothermia. If blood is not warmed before massive transfusion, ventricular arrhythmia and cardiac arrest may occur secondary to acute hypothermia. Blood may be warmed by passing it through a coil of tubing in a water bath maintained at less than 38ºC or with one of the commercially available blood warmers. The use of radio-frequency induction warmers to heat the entire blood bag has been associated with hemolysis.

(2) Bleeding diathesis. Another consequence of massive transfusion of stored blood may be the development of a bleeding diathesis, brought about by lack of platelets or certain coagulation factors. Platelets, although present in bank blood, are not very functional after 2 days of storage, while factors V and VIII have relatively short "in vitro" half-lives. Fresh-frozen plasma and/or platelet concentrates should be used for specific replacement therapy, when necessary.

(3) Hyperkalemia. During storage of blood, potassium slowly leaks from the cells into the plasma, increasing the plasma potassium concentration to about 17 mEg per liter by day 14 and about 21 mEg per liter by day 21. Although most recipients tolerate the potassium load without difficulty, patients with renal failure or those receiving exchange transfusion or other massive transfusion may develop acute hyperkalemia. This can be prevented by using blood less than 5 days old or by removing as much as possible of the potassium-rich plasma.

(4) Microemboli. A moderate amount of debris derived from platelets, fibrin, and leukocytes collects in bank blood during storage. Much of this particulate material is not filtered out by conventional 170-u blood administration set filters; this may cause pulmonary microemboli, leading to impaired oxygen transport ability in patients who receive large volumes of banked blood. Several filters capable of removing much of this material are commercially available and their use might be considered whenever the administration of large amounts of blood is anticipated.

3-10. DELAYED EFFECTS

a. **Hemolytic Transfusion Reactions.** Delayed hemolytic reactions occur, and usually result in extravascular removal of transfused cells from the circulation days to weeks following transfusion. Occasionally, abrupt intravascular hemolysis may occur with certain antibodies such as anti-JK^a, and anti-JK^b. Cells that were compatible at the time of infusion may be destroyed following antibody production. The direct antiglobulin test is usually positive, although the indirect antiglobulin test may be negative, until all transfused cells have been eliminated from the circulation. In general, delayed reactions, except for the very uncommon ones that produce intravascular hemolysis, tend to be asymptomatic and are only manifested by a mild, gradual anemIa and a transiently positive direct antlglobulin test.

b. **Viral Hepatitis.** The occasional occurrence of posttransfusion hepatitis remains a serious consequence of blood transfusion. Components such as plasma, platelets, cryoprecipitate, Factor VII I concentrate, factor IX concentrate, and fibrinogen are capable of transmitting hepatitis; the risk is proportional to the number of donors whose blood is used to prepare the component. Albumin, plasma protein fraction, and immunoglobulin preparations are regarded as safe derivatives since hepatitis virus is usually inactivated or removed during preparation. Tests for the hepatitis B surface antigen (HBsAg) have allowed detection of most carriers of hepatitis B virus (Section lll). Additional viruses capable of causing hepatitis can result in post-transfusion disease. In fact, most of the hepatitis now seen following transfusion with blood screened for HBsAg by radioimmunoassay or reversed passive hemagglutination techniques is not caused by either hepatitis A or hepatitis B virus. There is currently no known completely effective method for detecting the infectivity of <u>all</u> blood products capable of transmitting hepatitis. A system, for recording and reporting all cases of suspected post-transfusion hepatitis, is required.

c. **Autoimmune Deficiency Syndrome** In 1979, the Center for Disease Control (CCC) became aware of the increased incidence of an atypical form of Kaposi's sarcoma and of <u>Pneumocystis carinii</u> pneumonia. Investigation revealed the occurrence of lymphopenia, and a change in the ratio of helper to suppressor T-lymphocytes in these individuals. This combination of Kaposi's sarcoma and/or opportunistic infections with alterations in T-helper and T-suppressor lymphocytes has been called autoimmune seficiency syndrome (AIDS). One percent of AIDS cases have occurred in hemophiliacs who have been treated with large quantities of commercial Factor VIII concentrations. A case of AIDS in a neonate who received blood components from 19 donors raised the specter of single-donor transmission after one of the donors developed AIDS. The transmission of AIDS by blood transfusion is well documented. About two percent of AIDS cases are associated with blood transfusion. AABB, ARC, CCBC, and FDA have made recommendations to reduce the potential spread of AIDS through blood transfusion. These recommendations are as follows:

(1) Transfusion of blood and blood components should be given only for clear medical indications.

(2) Blood donors should be carefully screened and individuals in high-risk groups should be educated to abstain from donation.

(3) Autologous transfusion should be employed as widely as possible. The safety of the blood supply is fortified by a 4-point program:

(a) Voluntary blood donation.

(b) Careful medical history and physical examination to eliminate high-risk donors

(c) A sensitive test for anti-HIV.

(d) A confidential self-exclusion procedure.

d. **Other Diseases.** An infectious, mononucleosis-like syndrome characterized by splenomegaly, atypical lymphocytes, and fever, thought to be caused by cytomegalovirus infection, is occasionally seen following transfusions of large amounts of blood. Diseases such as malaria and syphilis can be transmitted by transfusion. The importance of the medical history in rejecting donors with unapparent malaria cannot be overestimated as there is no practical laboratory-screening test to detect donors with malaria. Since the treponemal spirochete does not survive 72-hour refrigeration, fresh blood or other blood products, such as platelet concentrates that are not stored refrigerated prior to use, have the greatest risk of syphilis transmission. The use of the RPR or other syphilis screening test lessens, but does not eliminate, the risk of syphilis transmission.

e. **Alloimmunization.** The transfusion of blood products always entails exposure to foreign antigens. Immunization to RBC, platelet, leukocyte, and protein antigens may occur to those that the patient lacks. Giblett has estimated the probability of stimulating one or more antibodies to RBC antigens after one blood transfusion to be about one percent.

3-11. RECORDS OF TRANSFUSION COMPLICATIONS

Federal regulations require that fatalities attributed to transfusion complications, for example, hemolytic reactions or viral hepatitis, and transfusion associated AIDS, be reported to the Bureau of Biologics of the Food and Drug Administration. In addition, records must be kept of reports of transfusion complications (including those investigated) and cases of transfusion-associated hepatitis (including those confirmed as type B) and transfusion associated AIDS for periodic reporting to this Bureau (see figures 3-1 and 3-2).

BLOOD TRANSFUSION REACTION REPORT

Clinical Diagnosis _____

Hospital Blood Unit Number _____ Amount of Blood Rec'd by Patient _____ ml.

Patient Identification

ATTENTION: Nursing Staff

1. Stop blood transfusion immediately, but leave needle in situ with slow saline drip.
2. Summon any available physician immediately to attend patient.
3. Check for agreement of all identifying names, numbers, and letters on pilot tubes, transfusion unit and patient's wrist tag.
4. Obtain an immediate posttransfusion clotted and anticoagulated blood specimen. Forward properly labeled specimen to Blood Bank. (Red- and lavender-stoppered tubes.)
5. Obtain and record pre- and posttransfusion vital signs below.
6. Obtain an immediate posttransfusion urine specimen and a second posttransfusion urine specimen 5 hours later. Forward properly labeled specimens to Blood Bank marked "transfusion reaction."
7. Request physician to complete box hereon marked: "Clinical Signs and Symptoms" with signature.
8. Send entire blood transfusion unit, posttransfusion clotted and oxalted tube, and urine specimen with this completed form to Blood Bank.

	Preresction	Reaction	Postreaction
Date			
Time	Blood started		Blood stopped
Temp.			
Blood pressure			
Pulse rate			

Temp. for 24 hours prior to transfusion	

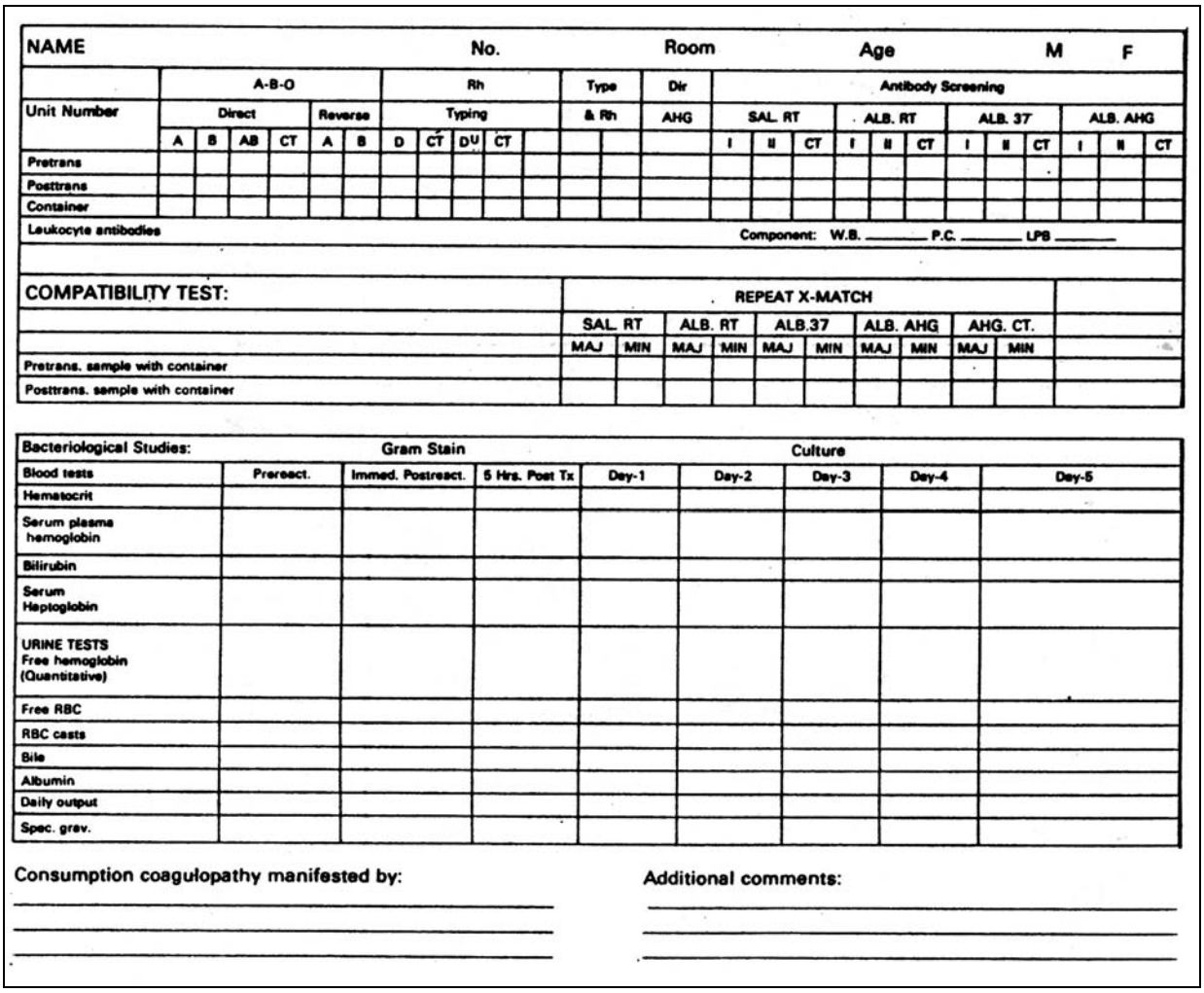
CLINICAL SIGNS AND SYMPTOMS:

Chilly sensation _____
Severe shaking chill _____
Severe low back pain _____
Hypertensive _____
Nausea _____ Arthralgia _____
Urticaria _____ Headache _____
Hematuria _____ Skin pallor _____
Perspiration _____ Dyspnea _____
Other _____ Oozing from wound or venipuncture _____

COMMENTS/CONCLUSIONS

Pathologist investigating M.D.

Figure 3-1. Blood transfusion reaction report.



NAME No. Room Age M F

Unit Number	A-B-O Direct A	B	AB	CT	A-B-O Reverse A	B	Rh Typing D	CT	DU	CT	Type & Rh		Dir AHG	Antibody Screening SAL. RT I	II	CT	ALB. RT I	II	CT	ALB. 37 I	II	CT	ALB. AHG I	II	CT
Pretrans																									
Posttrans																									
Container																									

Leukocyte antibodies Component: W.B. _____ P.C. _____ LPB _____

COMPATIBILITY TEST: REPEAT X-MATCH

	SAL. RT MAJ	MIN	ALB. RT MAJ	MIN	ALB.37 MAJ	MIN	ALB. AHG MAJ	MIN	AHG. CT. MAJ	MIN	
Pretrans. sample with container											
Posttrans. sample with container											

Bacteriological Studies: Blood tests	Gram Stain Preresct.	Immed. Postresct.	5 Hrs. Post Tx	Culture Day-1	Day-2	Day-3	Day-4	Day-5
Hematocrit								
Serum plasma hemoglobin								
Bilirubin								
Serum Heptoglobin								
URINE TESTS Free hemoglobin (Quantitative)								
Free RBC								
RBC casts								
Bile								
Albumin								
Daily output								
Spec. grev.								

Consumption coagulopathy manifested by:

Additional comments:

Figure 3-2. Investigation of transfusion reactions.

Section III. PREVENTION OF HEPATITIS

3-12. BACKGROUND

The incidence of hepatitis B associated with transfusion of blood and blood products has decreased significantly since the advent of methods of screening donors for the hepatitis B surface antigen (HBsAg); however, some risk of developing viral hepatitis after the transfusion of blood remains, even with the most sensitive currently available tests for detecting HBsAg, and with the use of volunteer blood donors. Measures designed to decrease further the incidence of post-transfusion hepatitis must be directed toward judicious utilization of blood, observation and follow-up of recipients of blood transfusion, exclusion of implicated blood donors, and further research into the etiology and pathogenesis of posttransfusion hepatitis C.

3-13. SELECTION OF DONORS

A review of some of the donor selection criteria is given in Lesson 1.

a. **History and Physical Examination.** Specific questions should be designed to detect a history of past or present hepatitis in the donor or close relatives (or other close contact with the disease), recent transfusion of blood components (or derivatives), recent tissue graft, and use of, or exposure to, potentially contaminated needles. The type of patient contact most hospital personnel have in their routine work is not considered "close contact" and such work is not a cause for donor exclusion; however, blood donors in close contact with individuals with viral hepatitis, for example, their spouses, should be excluded from donating blood for 6 months from the time of last exposure. Physical examination must include inspection of both arms for evidence of drug abuse.

b. **Payment of the Blood Donor.** It has been demonstrated that unpaid donors are to be preferred as there is no increased risk (5 to 15 times) of post transfusion hepatitis following the transfusion of blood from commercial source (paid donor) when compared to unpaid donors' blood. Payment for blood, per se, does not affect the quality of the blood of course, but this practice may attract undesirable donors, such as alcoholics or drug addicts, in whom the risk of hepatitis is high. In addition, the commercial donor is less likely to be reliable in terms of giving an accurate history of his present and past health.

3-14. TESTING FOR HEPATITIS B SURFACE ANTIGEN AND HEPATITIS C IN BLOOD

a. **General.**

(1) The AABB Standards require that all donor blood be tested for hepatitis B surface antigen (HBsAg) and hepatitis C (HCV). This is also a requirement of the Bureau of Biologics of the Food and Drug Administration (FDA). Hepatitis B surface antigen and HCV testing must use reagents and techniques specified by FDA or proven to have equivalent sensitivity and specificity. The FDA regulation states that all donor blood "shall be tested for the presence of the hepatitis B surface antigen by a method of sufficient sensitivity to detect all serums labeled A, (A), B, (B), and C in the Reference Hepatitis B Surface Antigen Panel, distributed by the Bureau of Biologics, except that, in emergency situations, a test method of sufficient sensitivity to detect all serums labeled A, (A), and B in the Reference Hepatitis B Surface Antigen Panel may be used." Thus, the most sensitive "third generation " test, such as radioimmunoassay (RIA) or reversed passive hemagglutination (RPHA), must be used for routine testing, while the less sensitive "second generation " tests, such as counterelectrophoresis (CEP) and latex agglutination, may be used in "emergency situations". The FDA regulation applies to blood or components for transfusion, to production of derivatives for transfusion, to manufacture of reagents for laboratory use, and to blood used for stimulation of antibody(ies) in donors.

(2) The results of HBsAg and HCV testing must be included on the label(s) of all units of blood and blood components, along with a statement of the possible presence of the agents(s) of viral hepatitis despite this testing being negative. Only in dire emergency situations may blood be transfused before completion of the HBsAg and HCV tests. If the test is subsequently found to be positive, the recipient's physician must be notified. Since each unit of blood must be tested at the time of donation, retesting for HBsAg and HCV are not considered necessary for blood from an "outside" source.

b. **Special Notes.**

(1) When RBCs are to be used for antibody stimulation, the HBsAg status of the cell donor should be negative at the time of each donation; when possible, a donor should be used whose blood is considered to carry a minimal risk of hepatitis.

(2) Do not routinely test intended transfusion recipients for HBsAg. HBsAg testing of the patient should be performed only when directly related to his diagnosis or care.

(3) The presence of anti-HBs in donor blood is not associated with an increased risk of type B hepatitis. Thus, testing for anti-HBs in donors is of little practical value at the present time.

(4) All HBsAg and/or HCV positive blood should be retested and confirmed as HBsAg-positive and/or HCV-positive since all test methods can give false positive results (see figure 3-3).

(5) As with all areas of the laboratory, quality assurance is important in HBsAg and HCV testing. Satisfactory participation in a proficiency test program should be part of the quality assurance of HBsAg tests (the American Association of Blood Banks and the College of American Pathologists sponsor such a program).

(6) All blood products and samples should be regarded as potentially infectious, whether are not they are HBsAg-positive. Minute amounts of HBsAg and associated hepatitis B virus may be undetectable in some serums by all current tests, yet the blood may transmit hepatitis B after inoculation or transfusion. In addition, HCV may be present and these are unrelated to HBsAg.

(7) The use of frozen RBCs (human) may diminish the risk of post-transfusion hepatitis. This effect, if substantiated, may be a result of the extensive washing of the thawed cells during de-glycerolization. The cost effectiveness of the routine use of frozen cells is questionable, so they should be used only when there is a clear-cut indication for their need.

Thank you for your recent participation in our blood donor program. Unfortunately, we cannot transfuse your blood because it may transmit viral hepatitis, an infectious disease causing liver inflammation, frequently accompanied by yellow jaundice.

In a continuing effort to decrease the frequency of post-transfusion hepatitis, we must test the blood of all donors for the presence of a substance which has been shown to be related to viral hepatitis type B. This substance-hepatitis B surface antigen (HBsAg) has been found in a number of donors throughout the United States since this screening test was initiated. Blood containing this antigen may produce hepatitis when transfused to a susceptible patient. On the other hand, the vast majority of donors who have this antigen show no evidence of having acute hepatitis or any other related medical abnormality. Thus, healthy donors may, nonetheless, transmit hepatitis to the recipient of their blood.

We are writing to you because this antigen was found in your blood at the time of your most recent donation. We would like to repeat this test on another sample to confirm our initial finding and, at the same time, perform additional tests for evidence of liver inflammation in you. This can all be done on a small (10 ml) blood sample. Would you kindly contact me to arrange a mutually convenient time for obtaining this sample? At the completion of these studies, I will explain our results to you and give them to your personal physician if you so desire.

If you truly are a carrier of HBsAg, this means that you should never again donate blood here or any other blood bank. While we appreciate your willingness to be a donor, we must consider the safety of the patients who would receive your blood.

We wish to thank you again for your blood donation and to stress that the finding of this antigen in your blood in no way diminished our sincere appreciation for your efforts in behalf of our patients.

Figure 3-3. Sample letter for notification of donors found to be HBsAg-positive.

3-15. RECIPIENT FOLLOW-UP FOR POST-TRANSFUSION HEPATITIS

a. It is imperative that the blood bank be notified of every patient who develops hepatitis, within 2 weeks to 12 months, following transfusion. In addition to notification by the recipient, his family, or his physicians, there are other sources for obtaining information about hepatitis patients:

(1) The hospital's medical record room.

(2) The gastroenterology service.

(3) The pathology department.

(4) The hospital's social service and business offices.

(5) The hospital's Infection Control Office.

(6) The local public health service.

b. All reported cases of acute liver dysfunction after transfusion of blood or blood components should be investigated as possible post-transfusion hepatitis, unless known to be otherwise. Sample forms for evaluating patients with possible post-transfusion hepatitis and for evaluation of implicated donors are provided (see figures 3-4, 3-5, 3-6, and 3-7).

c. A system for recording and reporting of all cases of known or suspected post-transfusion hepatitis is required by the AABB Standards. When a case of post-transfusion hepatitis is reported, all implicated donors must be identified and the facilities that collected the blood must be notified. It must be possible to trace a unit of blood and its components from final disposition (recipient, shipped, discarded) back to donor source and to recheck the laboratory records applying to the specific unit and its components and any adverse reactions or disease transmission.

d. Federal regulations require that fatalities attributed to transfusion complications (for example, hepatitis, hemolytic reactions,and so forth) be reported to the Bureau of Biologics. In addition, records must be kept of the number of cases of transfusion-associated hepatitis (including those confirmed as HBsAg-posltive) reported to the blood bank and the number of donors found to be HBsAg-positlve.

Attempts to reduce the incidence of viral hepatitis, an infectious disease that causes liver inflammation, have been extremely effective. The low hepatitis rate now associated with blood transfusions is due to the careful selection of donors and the testing of all donor blood for a hepatitis-related antigen (HBsAg). Despite these measures, however, an occasional case of post-transfusion hepatitis occurs. In such instances, as a final precautionary measure we recall all persons who have donated blood to the patient within the preceding B months. We do this so as to obtain an interim history on the donor and to perform certain laboratory tests related to hepatitis.

We are sending you this letter, because you were one of (number) donors to a patient who recently developed hepatitis. This should not alarm you, because having checked your blood once and found it to be normal; it is unlikely that we will find an abnormality at this time. However, this repeat check must be done for the ultimate protection of our patients. It is known that the majority of donors who transmit hepatitis are not themselves sick. The recipient of the blood may, however, become ill because he lacks resistance to the hepatitis virus.

In summary, this letter in no way implies that you were the donor to transmit hepatitis. Even If you were, it is unlikely that this has any implications in terms of your personal health.

We would, however, greatly appreciate your contacting the blood bank at your earliest convenience so that we may obtain a small blood sample (10 ml) and determine your interim medical history. Would you kindly contact:

_____
_____
_____

If any abnormalities are detected, you will be notified within 2 weeks. If you are not contacted, you may assume that all tests were negative and that you are still an eligible donor.

We greatly appreciate your cooperation in this matter. We especially appreciate your kindness in having been a blood donor.

Figure 3-4. Sample letter to recall donor implicated in post-transfusion hepatitis case.

You were one of many donors to a patient who recently developed post-transfusion hepatitis. Tests performed on your most recent sample show no evidence that you, yourself, have hepatitis. Tests on all of the other donors to this patient are also negative. When no donor can be specifically implicated, it is our policy to make a notation on the donor's card regarding this hepatitis involvement and to continue to allow future donations. If, however, such a donor is similarly involved on a second occasion, we request that he no longer donate blood for patients.

A review of our records indicates that this is the second time a recipient of your blood has developed hepatitis. This in no way implies that you were the one to transmit this disease since many other donors were also involved on each of these occasions. However, since our screening tests for hepatitis are not perfect and since we know that healthy donors can transmit hepatitis in their blood, we take the ultimate precaution of excluding anyone involved in 2 hepatitis cases. We realize that this is somewhat wasteful of donor resources and that it results in the exclusion of some persons who are perfectly safe donors, but we feel an obligation to our patients to do this.

In summary, we have found nothing in your blood to indicate that you have hepatitis, and there is no need for you to be concerned regarding your family. Your involvement in two cases of hepatitis may well be a chance occurrence due to your frequent donations. Nonetheless, in our current state of knowledge, we have no choice but to request that you do not again donate blood for patient care purposes (your blood may be used for research). It is hoped that more definitive tests for hepatitis will be developed in the near future. At such time, we may be able to pinpoint the implicated donor more precisely and clear the other donors, with more assurance. You may then, again, become an eligible donor.

I must emphasize our sincere appreciation for your previous donations and for your efforts in behalf of our patients. It is unfortunate that we must now exclude donors on such incomplete evidence. I hope that you will understand. I would be most happy to discuss this with your further, if you desire. I can be contacted at _____.

Thank you again.

Figure 3-5. Sample letter to donor implicated in two cases of post-transfusion hepatitis.

Date of Filling Out Report: _____

I. A. Patient's Name: _____

Birthdate: _____ Sex: _____

Address and Phone _____

B. Doctor at Time of Transfusion: _____

Address _____

C. Hospital and Chart No. at Time of Transfusion: _____

D. Diagnosis at Time of Transfusion: _____

E. Length of Hospital Stay: _____

F. Did Patient Receive Fibrinogen or Other Components? ___ Yes ___ No

II. A. Date of 1st Sign or Symptom of Hepatitis: _____

B. Description (Clinical Evidence of Hepatitis): _____

Laboratory Evidence of Hepatitis (including HBsAg test result): _____

_____

C. Additional History: other transfusion, contact with individual with hepatitis, drug addiction (needle-sharing), current medications, and so forth. _____

_____

D. Hospital at Time of Hepatitis: _____

Doctor at Time of Hepatitis: _____

Duration of Hospitalization: _____

E. Outcome: _____

1) Death: Date _____ Autopsy _____ Yes _____ No
Number: _____

2) Recovery: a) Partial--Date: _____
b) Complete--Date: _____

F. Donor numbers of implicated units: _____

_____
Name of Person Completing Form

Figure 3-6. Post-transfusion viral hepatitis history form.

Donor's Name: _____ Donor #: _____

Age: _____ Number of previous donations: _____

Previously implicated? ___ Yes ___ No

If yes, patient's name: _____ Hospital #: _____
Date of transfusion: _____

Results of donor's HBsAg test: Pos. __ Neg. __ Date _____ Method _____

Donor removed from active list: _____ 6 mos. _____Permanently

Letter of notification sent: ___ Yes ___ No _____ Date

Hepatitis patient (current recipient): _____
Hospital number.
_____

Duration of follow-up post-transfusion - Remarks: _____

_____

Review last four recipients within past 2 years of the above implicated donor of blood or blood components

Record of Follow-up Viral Hepatitis after Transfusion

Donation	Donor	Recipient Name & No.	Yes	No	Yes	No

Figure 3-7. Hepatitis implicated donor form.

3-16. HEPATITIS-IMPLICATED DONORS AND THOSE FOUND TO HAVE HEPATITIS B SURFACE ANTIGEN OR HEPATITIS C VIRUS

a. When a donor is implicated in a case of hepatitis, he should be recalled (see figure 3-4) and re-questioned in depth.

b. A blood sample should be drawn and examined for bilirubin, serum alanine aminotransferase (SGPT), HBsAg, and HCV. Abnormal results in any of these tests may exclude the donor from future donations. When hepatitis develops after exposure to multiple units of blood and/or blood components, it is not necessary to exclude all of the involved donors; but they should be noted as having been implicated once. A donor implicated in a 1-unit transfusion or a donor implicated in more than one case of post-transfusion hepatitis involving multiple units should be permanently excluded from future donation (see figure 3-5).

c. The excluded donor and the HBsAg-positive donor should be notified by the Medical Director of the blood bank by a letter (see sample letters), but personal contact prior to this is strongly recommended. The donor must be made clearly aware that he is exempted from future blood donations and that he has a potentially hazardous condition, but this should be handled with concern and tact. A repeat donor examination, follow-up laboratory tests, and personal discussion with the donor and his physician would constitute optimal management of persons who have been willing blood donors, but now must be permanently excluded from donating blood.

3-17. USE OF GAMMA GLOBULIN WITH TRANSFUSIONS

The routine administration of gamma globulin as either immune serum globulin (ISG) or hepatitis B immune globulin (HBIG) in all transfused patients to prevent viral hepatitis is not recommended. For patients accidentally exposed to blood products which are found in retrospect to be HBsAg positive, the use of ISG or HBIG should be considered.

3-18. LABORATORY SAFETY

In handling any human blood specimen, personnel must be aware of the possible risk of infection with hepatitis viruses. It is recommended that routine precautions be taken to protect the health of laboratory personnel. These include:

a. There should be no eating, drinking, or smoking in the laboratory.

b. Mouth pipetting is to be forbidden.

c. Personnel with open cuts or sores on their hands must not do HBsAg testing and must wear disposable gloves when handling specimens.

d. The HBsAg, HCV, HTLV-I, and so forth, testing should be done in a segregated area.

e. Hands should be washed before leaving the testing area (PVP iodine scrub is suggested). A sink in these laboratories should have a foot pedal, knee controls, or elbow blades.

f. Laboratory coats worn in the HBsAg testing area should be changed before returning to the general laboratory.

g. All test kits with positive controls and any blood that tests positive should be segregated if placed in refrigerators containing blood or blood products for transfusion.

h. The HBsAg, HCV, HTLV-I, and so forth positive material and disposal equipment used in testing for HBsAg should be placed in leakproof containers, labeled as infectious, and autoclaved or incinerated. Appropriate disposal methods must be used with all radioactive waste material. Consult the radiation safety official or committee of your institution for instructions regarding discarding radioactive materials.

i. Bench tops in test areas should be covered with nonabsorbent paper and be free of extraneous material (forms, reports, slips, and so forth.). General cleaning with bleach (1:5 dilution of hypochlorite) is recommended. Spills can be treated with cloth or paper material wetted with undiluted bleach. Liquid waste can be treated with equal volumes of hypochlorite, two percent formaldehyde, or other disinfectants suggested by the Center of Disease Control in Atlanta, Georgia.

j. Remember that positive controls and proficiency test samples are potentially infectious. Avoid entering vials with needles to prevent accidental parenteral exposure.

k. The HBsAg, HCV, HTLV-I positive blood, or secretions may be accidentally ingested by, or inoculated into, potentially susceptible individuals (those lacking anti-HBs). If so, prophylactic injections of immune serum globulin (ISG) or hepatitis B immune globulin (HBIG), if available, should be given. Careful follow-up for evidence of type B viral hepatitis for 9 months is advised.

Continue with Exercises

EXERCISES, LESSON 3

INSTRUCTIONS: Answer the following exercises by marking the lettered response that best answers the exercise, by completing the incomplete statement, by selecting true or false, or by writing the answer in the space provided at the end of the exercise.

After you have completed all the exercises, turn to "Solutions to Exercises at the end of the lesson and check your answers. For each exercise answered incorrectly, reread the material referenced with the solution.

1. Transfusions are most commonly given to restore:

   a. Nutrients and oxygen.

   b. White blood cells and antibodies.

   c. Immunity and platelets for clotting.

   d. Blood volume or RBCs for oxygen-carry.

2. Blood transfusions should be used only _____ since they may suppress erythropoiesis (production of red cells).

   a. In anemia.

   b. After surgery.

   c. As a last resort.

   d. When erythropoiesis is normal.

3. What is the product of choice for a patient with severe anemia requiring restoration of oxygen-carrying capacity?

   a. Platelets.

   b. Whole blood.

   c. Red blood cells.

   d. A plasma substitute.

4. The frequency and severity of leukocyte transfusion reactions is directly related to the number of incompatible leukocytes transferred.

    a. True.

    b. False.

5. What are the disadvantages of frozen red cells?

    a. Loss of cell viability and damage to proteins.

    b. Additional cost and limited storage period after thawing.

    c. Difficulties of running tests and of assessing viability.

    d. Lack of experience with an inadequate research of their use.

6. Why do the largest group of patients receive platelet transfusions when they have thrombocytopenia?

    a. Hemophilia.

    b. Massive blood loss.

    c. Chemotherapy for malignancy.

    d. Tissue damage requiring large amount of clotting.

7. ABO-compatible platelets are usually selected for platelet transfusion.

    a. True.

    b. False.

8. Rh-negative women of childbearing age without malignant disease should not receive platelet concentrates from _____ donors because of the effect of possible anti-Rh on future pregnancies.

    a. Rh-immune donors.

    b. Rh-positive donors.

    c. Rh-negative donors.

    d. ABO-incompatible donors.

9. What relatively high risk do commercial concentrates of Factor IX carry?

    a. Hemophilia.

    b. Hemolytic reactions.

    c. Disease transmission.

    d. Intravascular coagulation.

10. What is one advantage of commercial plasma substitutes (plasma protein fraction of 5 percent albumin in saline)?

    a. No risk of hepatitis.

    b. Usefulness in severe anemias.

    c. The presence of most clotting factors.

    d. Usefulness in congestive heart failure.

11. “Syringe” transfusions are recommended for premature and newborn infants.

    a. True.

    b. False.

12. What is removed by the filter used in the administration of RBCs, whole blood, and other products?

    a. Bacteria and viruses.

    b. Albumin and globulin.

    c. Leukocytes and platelets.

    d. Fibrin clots and other debris.

13. Because microaggregate filters are contraindicated in the transfusion of fresh whole blood, platelet concentrates, or granulocyte concentrates, they mostly remove:

    a. Platelets.

    b. Red cells.

    c. Granulocytes.

    d. All the above.

14. Which route should blood products be administered?

    a. Intravenous.

    b. Intradermal

    c. Intramuscular.

    d. Intra-arterial.

15. When the blood component is released from the blood bank, the technologists should check all of the following EXCEPT:

    a. Date of physician's request.

    b. Component and recipient numbers.

    c. Name of individual to whom the product is released.

    d. ABO group and Rh type of both the component and the recipient.

    e. Product name, both on the component and the blood request form.

16. What is the only fluid used for flushing and filling blood administration sets?

    a. Dextrose and Ringer's injection.

    b. Dextrose injection (5 percent in water).

    c. Dextrose and sodium chloride injection.

    d. Sodium chloride injection (normal saline).

17. How long should it normally take to administer a unit of whole RBCs?

    a. 15-30 minutes.

    b. 30-60 minutes.

    c. 1 to 1 1/2 hours.

    d. 1 1/2 to 2 hours.

18. Which item causes an adverse effect in blood transfusion because of compatibility-testing?

    a. Platelets.

    b. Leukocytes.

    c. Plasma proteins.

    d. Red blood cells.

19. Which specimen is needed to investigate suspected transfusion reactions?

    a. Post-reaction sample of recipient's urine.

    b. Pre- and post-reaction samples of the patient's blood.

    c. Blood from integral donor tubing or implicated container.

    d. All the above.

20. Whenever bacteria is suspected as the cause of transfusion reaction, it is necessary to perform a microscopic examination, and culture of the:

    a. Donor pilot sample.

    b. Recipient's pre-reaction blood sample.

    c. Recipient's post-reaction blood sample.

    d. Blood from the bag and from the patient.

21. Unpaid donors are preferable to paid or commercial donors.

    a. True.

    b. False.

22. It is required that all non-emergency donor blood be tested for HBsAg.

    a. True.

    b. False.

23. Hepatitis B is a highly contagious disease, which may be passed onto a recipient through transfusion.

    a. True.

    b. False.

24. Most transfusion recipients should be tested for HBsAg and HCV.

    a. True.

    b. False.

25. Anti-HBs in donor blood are a useful indicator of type B hepatitis.

    a. True.

    b. False.

26. All HBsAg and/or HCV positive blood should be retested and confirmed.

    a. True.

    b. False.

27. Special steps necessary to avoid hepatitis infection in the laboratory include:

    a. Avoid mouthing the pipette.

    b. Washing hands before leaving the laboratory.

    c. Wearing gloves, if open cuts or sores are on the hand.

    d. Segregating areas for: HBsAg, HCV, HTLV-I, and so forth, testing.

    e. All of the above.

Check Your Answers on Next Page

SOLUTIONS TO EXERCISES: LESSON 3

1. d (para 3-2a(1))
2. c (para 3-2a(2))
3. c (para 3-2b(2)(a))
4. a (para 3-2b(4))
5 b (para 3-2b(5))
6. c (para 3-3a(1))
7. a (para 3-3c(1))
8. b (para 3-3c(3))
9. c (para 3-4a(2)(b))
10. a (para 3-5a)
11. b (para 3-6b(2))
12. d (para 3-7b(1))
13. a (para 3-7c(2))
14. a (para 3-7d(1))
15. a (para 3-7e(2))
16. d (para 3-7f(2), (3))
17. d (para 3-7h(1))
18. d (para 3-8a)
19. d (Table 3-5)
20. d (para 3-9e(2))
21. a (para 3-13b)
22. a (para 3-14a(1), b(6))

23. a (para 3-14a(1))

24. b (para 3-14a(1))

25. b (para 3-14b(3))

26. a (para 3-14b(4))

27. e (para 3-18)

End of Lesson 3

APPENDIX

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End of Appendix